THE BIOLOGICAL AVAILABILITY OF COBALT TO RUMINANTS

by

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Declaration

I declare that the work submitted in this thesis was undertaken by me while at the Moredun Institue and that the assistance provided by others has been unconditionally acknowledged on the previous page.

Paul Graham McDonald, September 1983

Abstract

The aetiology of cobalt deficiency in ruminants is not clear and this study focussed on a crucial step in cobalt metabolism, namely its incororation, by rumen microbes, into the essential vitamin B12. Continuous cultures of rumen microbes (Rusitec) were used to study factors influencing vitamin B12 biosynthesis.

An improved technique for estimating cobalamin (true vitamin B12) in biological fluids rich in vitamin B12 analogues was developed.

Cobalamin and analogue production was increased, from the low levels encountered with cobalt deficient hays and barley, by infusing inorganic cobalt into the cultures. The utilisation of cobalt from cobalt deficient barley in the synthesis of cobalamin was more efficient than that from cobalt-deficient and cobalt-adequate hays, contrary to the generally held ideas. For any substrate, the synthesis of vitamin B12 analogues was far more efficient than that for cobalamin. Inorganic cobalt was incorporated into cobalamin and vitamin B12 analogues far less efficiently than that from either or hay and barley. Cobalt from four soils substantially increased cobalamin and particularly analogue synthesis. The use of monensin, a fermentation manipulator, had greater effects on digestibility and VFA production than on vitamin B12 synthesis.

CHAPTER 1

INTRODUCTION

The ability of ruminants to digest herbage extensively allows these animals to maintain production on land that might otherwise be unsuitable for farming. Domesticated ruminants are thus important, throughout the world, for the production of meat, milk, wool and associated products. Sub-optimum performances are not always recognised and for certain nutrients borderline deficiencies may be the most important economically.

Large areas of the world support herbage that provides low levels of certain trace elements. Among the primary causes for concern in Scotland is cobalt (Co). The high levels of sheep farming that occur in this country mean that studies of trace element utilisation are important and the recent report by the Council of the Scottish Agricultural Colleges (COSAC, 1982) highlighted the probability of feeds being deficient in Co, the regional variation in soil Co encountered and estimated that less than 50 % of the arable land in Scotland is reasonably adequately supplied with Co. Data on the economic importance of Co deficiency is scant, but Mills (1981) suggested that the incidence of this condition is not decreasing, its frequency of diagnosis between 1976 and 1979 placing it in the top 15 % of categorised diseases in the records of the Veterinary Investigation Diagnosis Analysis Service. The work described here was intended to help maximise the efficiency with which the ruminant utilises its Co intake under different dietary conditions.

LITERATURE REVIEW

The importance of cobalt to ruminants

In the development of pastoral systems throughout the world, certain areas were found to be unsatisfactory for the raising of sheep and cattle (Hogg, 1830), in spite of good pasture growth. Non-ruminants thrived in these areas, whereas sheep and cattle became weak and emaciated, progressively anaemic, and usually died. The disease was shown to be remedied by removal of the animals to "healthy" areas. These disorders became known by local names such as "bush sickness" in New Zealand, "pining" in Scotland and "salt sickness" in Florida, U.S.A. However, they were characterised by similar symptoms and were eventually classified under the term enzootic marasmus. For a period of several weeks or months the affected animals maintained apparent good health. This was followed by gradual loss of appetite, failure of growth or loss of body weight, succeeded by extreme inappetence, rapid muscular wasting (marasmus), depraved appetite (pica), and severe anaemia culminating in death. The severely affected animal was not unlike one which had been starved, except that the visible mucous membranes were blanched, the skin pale and fragile, and in sheep lachrymation occurred producing a dirty facial appearance (Underwood, 1966).

Early studies on this disease were undertaken at the end of the last century in the antipodes, where the economic

importance of this problem was greatest. Dosing sheep with soil from "healthy" areas was found to prevent this malaise (Aston, 1932a; 1932b; Rigg and Askew, 1934). Initially this was attributed to iron compounds in the soil because the anaemia of affected animals was corrected by dosing with iron compounds (Aston, 1932a; 1932b; Neal and Becker, 1933) and healthy areas had a high iron content (Greig et al., 1933). However, Rigg and Askew (1934) showed that supplying iron alone was not sufficient. Then in 1935, Underwood and Filmer proposed that a deficiency of dietary Co caused enzootic marasmus. They later substantiated this by showing that Co dosing acted as a curative and prophylactic agent (Filmer and Underwood, 1937). Further work confirmed it when low levels of Co were found in soil and herbage from "affected" areas, when compared to "healthy" areas, and that the livers of healthy sheep had higher levels than those of affected animals (Underwood and Harvey, 1938). This instigated studies into the dietary requirements of ruminants (McNaught, 1938), which themselves could be maintained by the development of aerial topdressing (Andrews and Pritchard, 1947).

Although the importance of Co was now appreciated, its role in animal nutrition was not understood. Tosic and Mitchell (1948) found that rumen microbes were capable of concentrating Co and that this accumulation accounted for up to 80 % of the total Co in the rumen when the diet contained less than 1.19 nmol Co/kg DM. Gall et al. (1949) were able to distinguish Co-adequate and Co-deficient sheep

on the basis of their microflora, numbers and types. Such information, allied to the known immunity of non-ruminants on deficient areas, suggested that Co acted in association with the rumen.

Ray et al. (1948) showed that orally administered Co was considerably more effective than parental Co in the remission of Co-deficient sheep. The efficiency of oral dosing was soon confirmed (Marston and Lee, 1949; Smith et al., 1950; Keener et al., 1951). The rumen was shown to be the major site of action for Co by Phillipson and Mitchell (1952), and later Lee and Marston (1969), who introduced Co into the duodenum with no effect. Responses to exremely large parenteral and duodenal doses of Co were explained by the excretion of small but significant amounts in the saliva. Early distribution studies (Comar et al., 1946; Comar and Davis, 1947) using radioactive Co had found that such secretions occurred.

The reason why the passage of Co through the rumen should be so important became clear as a result of quite separate investigations of pernicious anaemia in man.

Isolation of vitamin B12 and its relation to animal protein factor

Pernicious anaemia (p.a.) is an established, fatal disease of man (Combe, 1824). In 1926, Minot and Murphy demonstrated the efficiency of ingested liver in treating p.a. Concentration of the anti-pernicious anaemia factor

(a.p.a.f.) was attempted and liver extracts were produced commercially. Then in 1948, 2 groups reported the successful isolation from liver of a Co-containing compound, vitamin B12 (Rickes et al., 1948a; Smith and Parker., 1948). Its therapeutic value for p.a. patients was demonstrated by West in the same year.

Prior to the isolation of vitamin B12, liver extracts had been grouped with other animal "concentrates" which were thought to contain the animal protein factor (a.p.f.). This was the name given to those animal extracts shown to be necessary for the optimum growth of rats, chicks and pigs fed on vegetable rations. When vitamin B12 was isolated it soon became identified with a.p.f., as fermentation products known to contain a.p.f. had been shown to be effective in p.a. (Stokstad et al., 1948). The difference in response between pure vitamin B12 and a.p.f., when tested by microbial and chick assays, demonstrated that a.p.f. contained more than vitamin B12 (Coates et al., 1951b). The extra components have been suggested as being some antibiotics and essential amino acids (Friesecke, 1981).

Large oral doses of liver had been fed to sheep many years before and had cured Co-deficiency in some (Filmer, 1933), but not all instances (Marston and Lee, 1952). Filmer and Underwood (1937) proposed "that the potency of liver may be due to the presence of a stored factor and that Co may function through the production of this factor within the

body". The isolation of vitamin B12 and the presence of Co as a constituent (Rickes et al., 1948b) led to the proposal that ingested Co was responsible for the production of the vitamin in the rumen (Becker et al., 1949). Hale et al. (1950) found a positive correlation between Co intake and the vitamin B12 level in rumen contents and vitamin B12 compounds (p 7) were found to be more abundant in the rumen contents of Co-supplemented than Co-deficient animals (Hoekstra et al., 1952a).

Armed with the hypothesis that Co deficiency was primarily a deficiency in vitamin B12, attempts were made to achieve remission of the condition with the vitamin. Early studies with oral (Becker et al., 1949; Becker and Smith, 1951a; Marston, 1952) and parenteral vitamin B12 (Becker et al., 1949; Marston and Lee; 1949; Becker and Smith, 1951a) were ineffective. However, some injected liver extracts were shown to produce a positive response (Becker and Smith, 1951a; Smith et al., 1951b). Later work showed increased amounts of injected vitamin B12 to be effective (Anderson and Andrews, 1952; Hoekstra et al., 1952b; Marston and Lee, 1952; Marston and Smith, 1952). Oral vitamin B12 was subsequentley shown to cure Co deficiency if supplied in sufficient quantity (Kercher and Smith, 1955).

With the realisation that vitamin B12 was one of a group of related compounds, whose biological activity varied in different microorganisms (Ford et al., 1951; Ford, 1952), investigations were undertaken to test for their

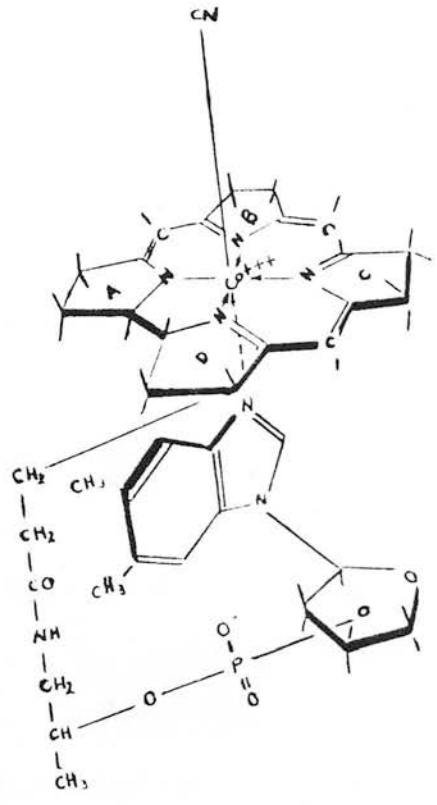
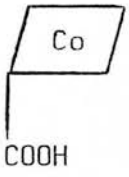
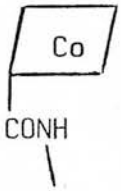
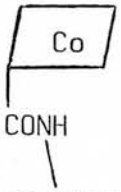


FIG. 1.1 Structure of cyanocobalamin.

TABLE 1.1 Nomenclature of the corrinoids.

The f-carboxyl is that group on the D ring to which is attached the Co_{α}-nucleotide; the remaining carboxyl groups are attached around the edge of the corrin ring.

Substituent on f-carboxyl	Name		Abbreviation (for the hexamide)
	Other carboxyl groups unsubstituted	Other carboxyl groups amidated	
	Cobyrinic acid	Cobyric acid	Cby
	Cobinic acid	Cobinamide	Cbi
	Cobamic acid	Cobamide	Cba
As above, with base = 5,6-dimethylbenzimidazole	-	Cobalamin	Cbl

therapeutic value. One of these, vitamin B12a (hydroxocobalamin), was found to be as effective as vitamin B12 (Koch and Smith, 1951), while another, pseudovitamin B12 ((adenyl) cobamide) was shown to be ineffective in both lambs (Kercher, 1954, cited by Smith and Loosli, 1954) and calves (Hopper and Johnson, 1955).

Structure and nomenclature of vitamin B12 and related compounds

The only known function of Co in animal nutrition is as a component of vitamin B12 and analogues (Underwood, 1966). Elucidation of the structures showed them all to be members of the corrinoid group of compounds. The structure of true vitamin B12 (cyanocobalamin) is shown in Fig. 1.1. "True" refers to the fact that the cobalamin (Cbl) class of corrinoids is the only form known to be physiologically active in mammals. Recommended nomenclature is that of the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) (1973), as summarised in Table 1.1. This will be adopted throughout, but in abbreviated form, when specific names are used,

e.g. common name is true vitamin B12

correct name is Co α-[α-(5,6 dimethylbenzimidazole)]

-Coβ -cyanocobamide

abbreviated name is cyanocobalamin (CN-Cbl)

The 2 coenzymic forms of Cbl known to function in mammalian metabolism are 5'-deoxy-5'-adenosyl cobalamin

(adenosyl-Cbl) and methylcobalamin (methyl-Cbl) (Grasbeck and Salonen, 1976). CN-Cbl is in low concentrations physiologically and is to a large extent an artefact due to the isolation procedure, but it is the most stable derivative of the Cbl family (Grasbeck and Salonen, 1976). Further use of the term true vitamin B12 will refer to the Cbls, a subgroup of the corrinoids; vitamin B12 is used on the few occasions when quoting references in which the form of the vitamin measured is not revealed. The term total vitamin B12 refers to Cbl plus analogues, when analogues comprise forms of the corrins other than Cbl. In references mentioning total vitamin B12 the analogues measured will be dependent upon the analytical technique used (p 55). In referring to blood, total vitamin B12 values will approximate to Cbl as the occurrence of analogues in blood is limited; however, their influence may be significant.

Cobalt in soil

For grazing ruminants, the majority of nutrients are provided by the plants they ingest. The Co content of the herbage is dependent on the soil upon which it grows. Animals suffer from Co deficiency primarily because of the lack of "plant-available" Co in the soil (Underwood, 1966). Co uptake by plants is dependent upon 2 factors; the Co content of the soil and the "availability" of this soil Co to plants.

Co occurs in the earth's crust at 40 mg/kg (679 µmol/kg), so defining it as a geological trace element (Latteur, 1962). The level of the element in soil is determined by the nature of its parent rock (West, 1981). Typically, basic and ultrabasic igneous, e.g. gabbro, have a higher concentration of Co than acidic igneous rocks, e.g. granite, (West, 1981). Pedological and biological weathering to produce the agriculturally important, sedimentary rocks will influence the Co content of the derived soils.

Determination of total Co requires the destruction of organic matter prior to analysis (Young, 1966) and levels of total Co up to 6.16 mmol/kg have been found in the sedimentary limonite of New Zealand (Latteur, 1962) and up to 17.0 mmol/kg in areas of the world rich in minerals (Young, 1979). Plant-available Co is that which can be extracted into acetic acid or other solutions (West,1981); the extractant will determine the form(s) of Co measured. Those forms of the element in soil solution will comprise the plant-available fraction (West, 1981). Loneragan (1975) cited the proportion of complexed Co in soil solution to be 8 to 50 %. In acidic soil the dominant inorganic Co ion is Co^{2+}, while in neutral and alkaline soil the hydroxy ion $Co(OH)^{+}$, would also be important (Lindsay, 1972). Levels of plant-available Co up to 115 µmol/kg have been determined. This available Co normally constitutes 3-20 % of the total Co, but it can be as much as 90 % (Young, 1979).

Many factors influence the level of Co available to the plant (see below). Determinations of total or plant-available Co suffer from the lack of a suitable standard (Beckwith, 1963). Poor correlation between the increases in extractable (available) soil Co and those in herbage Co, for Co additions to 4 deficient soils, caused McLaren et al. (1979) to question the value of this soil Co measurement, although Davies and Crawshaw (1978) found low available Co levels produced an adequate herbage Co status. Reliance on total Co values has, in some instances, also been found to be illusory (Poole et al., 1974). COSAC (1982) advised the use of total Co values in the B (subsoil) horizon to give a measure of soil reserves and approximate plant availability; these figures to be used in conjunction with the previously mentioned influencing factors in classifying a soil. However, they stressed the preliminary nature of this exercise.

Acidic soils favour the absorption of Co by plants (Mitchell, 1957; Reith and Mitchell, 1964). Adams et al. (1969), using pot cultured plants, found that a reduction of soil pH from 6 to 4 increased the herbage content 10-fold, for both Co fertilised and unfertilised soils, albeit with a reduced plant yield in most instances. Reductions in the pH of the soil have been shown to occur during the growing season (Whinham, 1979) and might have been responsible for the increase of Co in mixed herbage with successive cuts (Reith et al., 1979). Conversely, liming

of the soil reduces the available Co content (Hill et al., 1953; Reith and Mitchell, 1964); probably by converting Co to its less soluble carbonate salt (Banerjee et al., 1953). The use of nitrogen fertilizers lowered extractable soil Co and herbage Co (Reith et al., 1979), an effect that is greatly reduced on poorly drained soils (COSAC, 1982). Waterlogging of the soil has been shown to increase available soil Co and plant Co levels (Hill et al., 1953; Adams and Honeysett, 1964). Hill et al. (1953) found an inverse relationship between total soil Co and soil particle size and Tiller et al. (1969) showed total Co to be correlated with the soil surface area. Total Co content has been found to increase (Hill et al., 1953) and decrease (Ozanne et al., 1963) with depth, while Forbes (1976) suggested that topdressed Co elevated Co levels in the upper 7.5 cm of soil substantially. A separate study showed that available Co, as measured by 3 extractants, tended to decrease with depth (Berrow and Mitchell, 1980).

Specific soil interactions are known to occur with Co. Iron oxides, organic matter and clay can all adsorb Co (McLaren et al., 1979), restricting its availability to plants, but the most important single factor is considered to be manganese oxide (Adams et al., 1969; McKenzie, 1975). It has been shown to possess a strong, specific affinity for Co (McKenzie, 1975). This binding may explain the increased availability of Co in waterlogged soils, since manganese complexes are unstable under these conditions

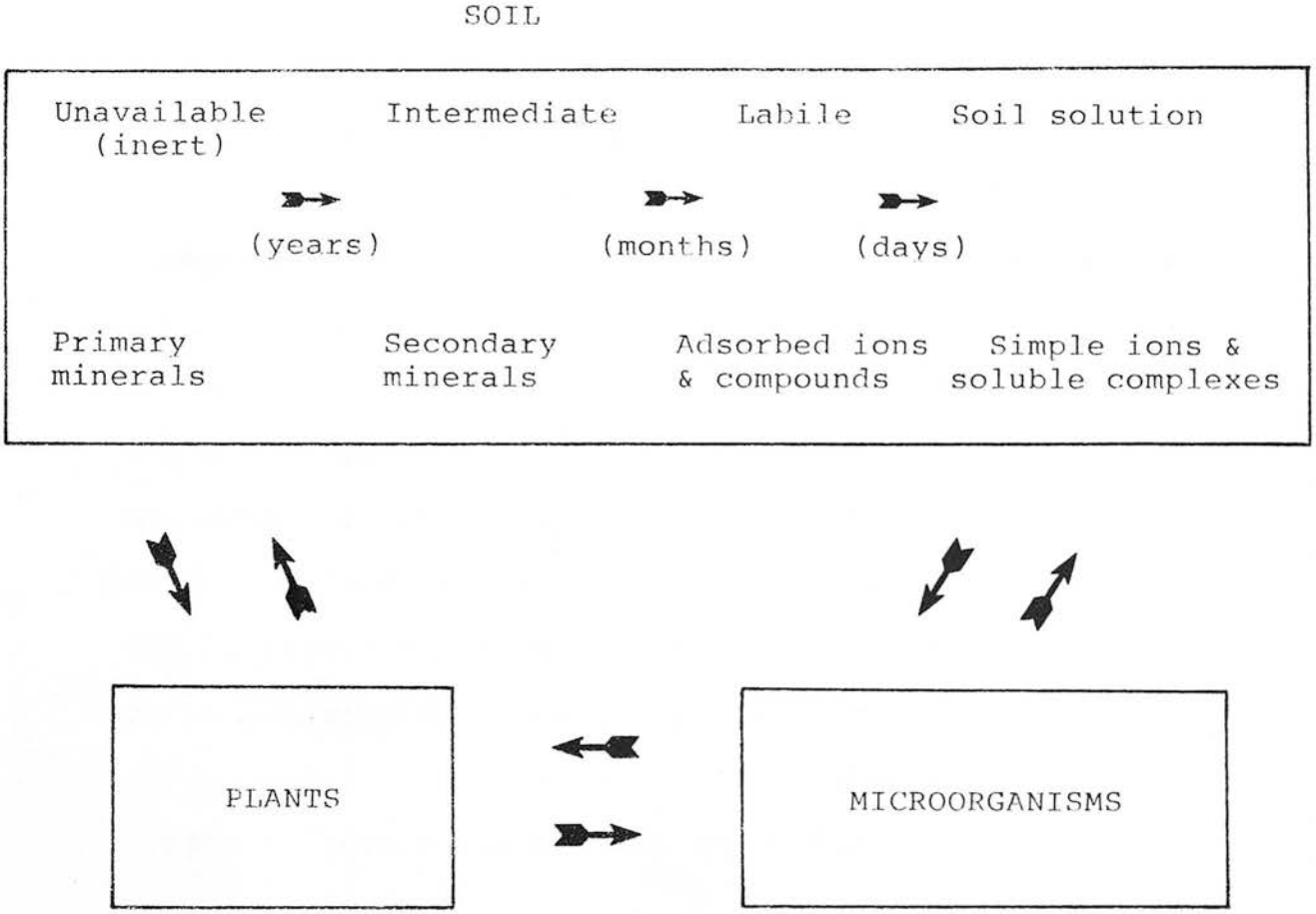


FIG. 1.2 Forms of cobalt in soil and their relationship to plants and microorganisms.

(Adams et al., 1969). Microbial oxidation of manganese to the oxide can occur and this may be more influential than physico-chemical factors in oxidation (Loneragan, 1975, Nambiar, 1975). Waterlogging will influence the microbial populations by producing anaerobic conditions. It has been suggested that atmospheric depositions on agricultural land can contribute up to twice the Co content of a herbage crop (COSAC, 1982).

The forms of Co in soil, their equilibria and relationships with other soil factors are summarised in Fig. 1.2. Co will exist as free ions, chelated and complexed Co in true or colloidal solution (Ermolenko, 1972; Loneragan, 1975; West, 1981). The majority of Co is not in solution, so ensuring that the soil is not depleted by leaching (West, 1981).

Soil/plant relationships

Absorption of Co can be considered in 3 stages (Loneragan, 1975).

1. The supply of Co into solution (available Co) has been discussed previously.

2. Absorption by the plant is related to the concentration of Co, the ionic millieu and interactions with other ions (Loneragan, 1975; Tinker, 1981). It is not known whether absorption is an active or passive process, if the form of Co is crucial, e.g. ionic, chelated, or whether there are differences between plant species in uptake (Loneragan, 1975; Tinker, 1981).

3. Soil-plant interactions occur that influence the soil, the plant, and ultimately the absorption of trace elements; examples of these are

a) root secretions that influence soil and microbial composition and possibly the form of Co in solution (Ermolenko, 1970; Loneragan, 1975; Tinker, 1981),

b) compacted soil structures in established pastures which may restrict leaching. Modification and redistribution of available Co in the soil (Thornton, 1979) will effect the amount of Co available to the plant, as determined by the rooting zones.

Such factors provide further reasons why soil Co values can only be an approximate indicator of plant Co status.

Cobalt in plants

Species differences Different plant species have been found to assimilate different amounts of Co (Thomas et al., 1952; Hill et al., 1953; Mitchell, 1957; Andrews, 1971). Levels are normally highest in herbs, legumes, cereal forages and weeds, with lower levels in mature cereals, grasses and the subterranean fraction of vegetables. Andrews (1971) found different pasture plants grown under the same conditions to have the Co levels shown below;

Plant	Cobalt(µmol/kg DM)
timothy	1.53
cocksfoot	1.87
meadow fescue	2.04
short rotation ryegrass	2.21
perennial ryegrass	2.72
red clover	3.91
white clover	4.08

While certain species, e.g. Astragalus, may contain up to 1.70 mmol/kg DM, the values for pasture species are normally up to 6.79 µmol/kg DM (Young, 1979). On Co-deficient soils, species differences decrease considerably (Andrews, 1966). Under such conditions, West (1981) found the uptake by grass to be superior to that of clover.

Intra-specific variation occurs (Beeson et al., 1947), and may be important when a single species dominates a pasture. Pasture composition may encourage or depress the growth of plants with high Co levels.

Maturity/stage of growth The growth of a plant affects its mineral content. In most circumstances the Co level declines as the plant matures (Underwood, 1966; Tinker, 1981). However, Adams et al. (1969) found a tendency for the reverse to occur and Reith et al. (1979) found that the Co content of successive cuts increased throughout the growing season. These variations will themselves be

influenced, if not determined, by climatic changes during the growing season, e.g. a spell of excessive rainfall affecting growth rate and soil Co availability.

Improvement of hill land has also been found to increase the risk of Co deficiency (COSAC, 1982). This is partly due to liming, but also to the introduction and encouragement of particular plant species with faster growth rates which produce herbage of lower Co content.

Distribution within plants Within a plant, distribution of Co is not uniform. Latteur (1962) found the greatest concentration in the leaves and seeds, less in the fruiting and rooting organs and very little in the shells and kernels, after meaning results from a range of cultivars. In contrast, Underwood (1966) stated that the bran was a good source of Co. Handreck and Riceman (1969) using pot cultures of pasture species found Co concentrations to be maximal in the leaf margins for some species and at the base and tips of others; the greatest levels being in the youngest leaves. Leaching of Co can cause substantial losses (up to 75 %) in dried leaves, a phenomenon that may be important in hay production.

Soil contamination and seasonal variation The amount of Co associated with pasture tends to increase in late autumn and winter and to decrease in spring and summer (Andrews, 1971). This may be due to the increased soil contamination ensuring a greater Co intake by grazing animals when

pastures are short and so it contributes to the seasonal occurrence of ill-thrift. For marginal or deficient herbage, variations in the level of Co due to season and/or stage of growth may be sufficient to produce or alleviate deficiency symptoms in grazing ruminants (Lee, 1950; Andrews, 1971).

Cobalt requirement of plants

Co is important to plant as well as to animal life, being required for nitrogen fixation in free-living bacteria, blue-green algae and symbiotic rhizobia (Ahmed and Evans, 1959; Nicholas et al., 1962; Evans et al., 1965, cited by Nicholas, 1975). In root nodules Co is thought to be required for the production of cobamide compounds by the rhizobia (Nicholas, 1975). Co ions are also known to activate some carboxylases and peptidases in plants (Meyer et al., 1968). A deficiency for Co has been exhibited by legumes in field trials (Powrie, 1961; Ozanne et al., 1963). The level of Co in the leaves of deficient plants was less than 679 nmol/kg DM (Ozanne et al., 1963), but the level of Co needed to sustain optimum plant growth may not necessarily be that required by animals. Water-culture experiments have shown the toxicity of Co to plants at levels as low as 136 µmol/l for maize, 102 µmol/l for wheat and rye and 170 µmol/l for barley and peas (Latteur, 1962). These levels of Co may influence iron metabolism (Latteur, 1962) or the physiologically important production of ethylene (Yu and Yang, 1979).

Form of cobalt in plants

Little is known about the form of the element in plants. Wiersma and Van Goor (1979) found Co in phloem sap to be complexed, possibly to polynucleotides. Translocation of Co through the plant is thought to occur as the soluble form, either ionic or in a small molecular weight complex (Tinker, 1981). Storage occurs as a complex with plant carbohydrates or proteins (Tinker, 1981).

Plant/animal relationships

In certain areas, sheep, and to a lesser extent cattle, grazing pastures containing some perennial grasses of the Phalaris genus develop a condition called "phalaris staggers". It is so called because it produces neurological disorders that can become permanent and may eventually be fatal. Orally administered Co was found to produce remission of the condition (Lee and Kuchel, 1953). The site of action is thought to be in the rumen, but its mode of action does not depend upon the production of Cbl (Lee et al., 1957). That the condition does not always develop in animals grazing "Phalaris pastures" is considered to be due to there being a sufficient dietary Co intake, for the prevention of degenerate neurological changes. The agent responsible for the condition and the mode of action of Co remain unknown.

Selective grazing may deplete a pasture of the Co-rich species such as legumes. Stocking rates will influence the

length of the pasture and with it soil intake as a dietary contaminant. Grazing pressure also affects the amount of new growth and thus the Co content of the herbage, since new growth tends to have a higher Co level than mature herbage (Handreck and Riceman, 1969). Understocking can lower Co intake by allowing more plants to mature to a relatively low Co state; furthermore, a low digestibility may give rise to a reduced dry matter (and hence Co) intake. Variations in dietary Co intake are reflected by wide variations in serum Cbl levels (p 39).

Criteria of deficiency for cobalt in soils, pastures and diets

The need for preventative measures can be anticipated to some extent by Co analysis of soil and herbage. Because of the many interactions mentioned previously the soil Co level above which a satisfactory dietary intake will be maintained is a debatable figure. 5 p.p.m. (84.9 μmol/kg DM) has been quoted as the acceptable level for total Co in soil (Poole et al., 1974) and 0.3 p.p.m. (5.09 μmol/kg DM) for extractable (plant-available) Co (McLaren et al., 1979); although many other values have been suggested (Young, 1979). Soil values have been used, in conjunction with animal Cbl content, to define marginal, moderate and severely deficient regions in New Zealand (Andrews, 1971). A recent report (COSAC, 1982), classified air-dried soils by the level of extractable soil Co when using 0.43 M acetic acid.

Soil status	Soil Co (µmol/kg)	Herbage Co (µmol/kg DM)
deficient	<5.09	<1.36
borderline	5.09-6.79	1.36-1.70
adequate	>6.79	>1.70

The critical values are adjusted when the pH of the soil is appreciably different from 6.0 and for soils with total organic matter above 15 %.

In view of the previously mentioned influences on plant Co, soil values have a limited use and consideration of herbage levels is preferable as a diagnostic aid.

Dietary cobalt requirements

Investigations into the minimum dietary requirement for Co showed that regular dosing with small amounts of Co was more effective than less frequent, larger doses (Lee, 1950; Andrews et al., 1966); 119 µmol Co once a week for 5 weeks being more effective than 594 µmol every 5 weeks (Lee, 1950). The minimum Co requirements, which have been estimated by many workers (Filmer and Underwood, 1937; McNaught, 1938; Marston, 1952; Lee and Marston, 1969; Somers and Gawthorne, 1969; Marston, 1970), cover the range 1.36-2.21 µmol Co/kg DM for pasture and semi-synthetic diets.

The A.R.C (1980) recommended 1.87 µmol Co/kg DM (0.11 mg/kg Co DM) as the minimum requirement for pasture or conserved roughage, but suggested that this level may not be adequate for high metabolisable energy diets.

Manipulation of cobalt concentrations in the diet

Pasture levels can be improved by the addition of Co salts in fertiliser or their application as a foliar spray, a method introduced by Andrews and Pritchard (1947). Current recommended levels for Scotland (COSAC, 1982) are 2 kg $CoSO_4$ /hectare, repeated every 4 years. For severely deficient land this application should be increased 3-fold. Carbonate, phosphate and sulphate anions have been shown to produce equivalent results (Askew and Watson, 1946). West (1981) advised against the use of Co EDTA in soil additives on both efficacious and economic grounds; although the use of chelated trace elements in applications to both pasture and animals is common. The work of McLaren et al. (1979) in S.E. Scotland illustrated the failure of Co applications to pasture on certain soils and the need for deficient areas to be carefully evaluated. Co may also be provided via salt licks or free-access minerals to both stall fed and grazing animals.

When animals are housed and/or fed supplements Co can be added to the rations. Alternatively, piped water supplies can be treated using a concentrated Co solution (3.40-6.80 μmol Co/kg H_2O) from a flow meter plumbed into the water supply (COSAC, 1982).

Variable consumption is a problem inherent in all these treatments. In most instances, pasture applications of $CoSO_4$ will be the most economic solution (COSAC 1982).

Cobalt intake of ruminants

For housed animals, the intake of Co will be that provided by the diet plus metallic contamination from the licking and/or chewing of housing materials. Levels in plant material have been discussed previously. The drinking water is rarely a significant source of Co, most fresh water values being within the range 1.70-170 nmol Co/Kg H_2O (Young,1979), but notable exceptions are the high levels in some spring, mineral and mine waters.

Grazing animals have an additional source of Co, namely the soil which contaminates herbage and crops. Soil ingestion is maximal on short, winter pastures, when pastures are overgrazed or when root crops are used (Healy, 1967; 1968; Healy and Drew, 1970). The possible importance of fortuitous ingestion of trace elements in soil has been recognised (Field and Purves, 1964; Healy et al., 1970; 1974; Fleming, 1975). For animals grazing winter pasture it has been estimated that soil may provide upto 35 times more Co than pasture (Healy, 1967). The biological availablity of this Co may vary between soils (MacPherson et al., 1978). Contamination of well grazed pastures with faeces will provide localised areas of Co-rich herbage, as estimates of the amount of orally administered Co voided in the faeces have ranged from 65-95 % (Comar et al., 1946; Comar and Davies, 1947; Monroe et al., 1952; Smith and Marston, 1970a), although much of this will be as vitamin B12.

The chemical states in which Co enters the rumen are therefore varied and will be the plant and soil forms mentioned previously. In addition to these, there will be traces of endogenous Co that are recycled into the rumen via the saliva (Comar *et al.*, 1946) and the rumen wall (Grace, 1975). Small amounts of Cbl and analogues may be ingested with feed, either through the diet, e.g. animal protein sources, or as a result of microbial synthesis. Other small sources are soil and silage associated microorganisms. Grzeszczak-Swietlikowska (1964) found that additions of Co, 5,6-dimethylbenzimidazole and *Propionibacterium shermannii* (a known vitamin B12 producer) could transform alfalfa silage into a notable contributor of the vitamin. Faecal (Ford and Porter, 1953; Dawbarn and Hine, 1955) and urinal (Pearson *et al.*, 1953; Dawbarn and Hine, 1955; Teeri *et al.*, 1955) contamination of the feed may provide major and minor contributions respectively, to the dietary vitamin intake. However, the vast majority of the animal's needs for Cbl must be catered for by ruminal synthesis using ingested Co.

Cobalt toxicity

In vitro rumen studies indicated that a Co level in excess of 204 µmol/l in the incubation medium resulted in depressed microbial activity (Salsbury *et al.*, 1956; Hubbert *et al.*, 1958; Martinez and Church, 1970). In contrast, Streeter (1961, cited by Martinez, 1972) found *in vitro*, that levels between 170 and 849 µmol/l proved

stimulatory to cellulose digestion. Co inhibition of *in vitro* cellulose digestion at a level of 42.5 µmol/l could be partially ameliorated by the inclusion of magnesium at 10.3 mmol/l (Martinez, 1972).

At high levels Co has been found to be deleterious to ruminants. While daily doses of 50.9 µmol Co/kg l.w. can be tolerated by sheep, supplements of 170 µmol Co/kg l.w./d depressed appetite and body weight, produced anaemia and caused death (Becker and Smith, 1951b). This anaemia may be associated with a depressed iron absorption caused by an increased Co intake (Underwood,1977). Non-fatal chronic toxicities in sheep may occur at Co intakes of 17-170 µmol Co/d and liver Co levels of 17.0-170 µmol/kg DM (Andrews, 1965). Young dairy cattle were found to tolerate upto 849 µmol Co/45 kg l.w/d without ill-effect (Keener *et al.*, 1949); suggesting that they are more tolerant of Co than sheep. Levels of liver Co in cattle assumed to have died from Co toxicity ranged from 84.9-4890 µmol/kg DM (McLaren *et al.*, 1964; Dickson and Bond, 1974). The wide margin between the safe and toxic levels for Co in ruminants is unlike other elements, such as copper and selenium.

Ruminal cobalamin and analogue production

Extracellular, ruminal digestion would be expected to release some, if not all, of the plant and soil Co ingested. Reducing conditions favouring the breakdown of manganese oxide (Nambiar, 1975), as would be expected in the rumen, should aid liberation of soil Co. The levels of

Co producing a favourable effect on ruminal activities, e.g. VFA production, protein synthesis, have been reviewed by Durand and Kawashima (1980). They suggested an optimum level of Co in the rumen fluid of 1.70-8.50 µmol Co/l, which required a dietary level of 8.50-17.0 µmol Co/kg DM. Rumen bacteria have been found to have a requirement for Co in excess of 11.9 µmol Co/l culture media (Mori and Kandatsu, 1975). However, Smith and Marston (1970b) found that the digestibility in a rumen depleted of Co was not affected by Co supplementation. Of the studies undertaken on ruminal Co the vast majority have been concerned with Cbl and analogue production and it is these aspects that will be discussed.

Co is only known to be required by rumen microbes and the host animal as a constituent of Cbl and its analogues (Underwood, 1966). It may be through these compounds that effects on other bacterial activities are mediated. Gawthorne (1969) separated 9 forms of the vitamin from rumen fluid, after early work had shown the presence of analogues in the rumen (Ford et al., 1953b; Dawbarn et al., 1957a). The production of these analogues is influenced by a number of factors.

Cobalt Increasing Co intake increases the total vitamin B12 content of the rumen (Hale et al., 1950; Hoekstra et al., 1952a; Hine and Dawbarn, 1954; Hedrich et al., 1973) and in vitro work (Gawthorne, 1970b) confirmed that Co was the primary limiting factor. Levels of vitamin B12 production

TABLE 1.2 Effects of the proportion of roughage and the cobalt intake of the diet on the percentage of ingested cobalt incorporated into cobalamin (conversion efficiency) by rumen microbes in sheep.

Diet (% roughage)	Co intake (µmol/d)	Cbl production (nmol/d)	Conversion efficiency (%)	Ref.
94	0.713	81.2	11.4	1
94	17.7	539	3.05	
100	29.2	1638	5.61	2
0	19.7	566	2.87	
100	13.3	882	6.64	3
70	11.0	786	7.20	
40	9.19	445	4.82	
69	0.798	27.3	3.42	4
69	6.96	742	10.7	
69	14.1	1146	8.13	
70	9.49	790	8.33	5
70	11.4	1690	14.8	
39	9.69	831	8.57	

References: 1= Smith and Marston (1970), Co as oral dose at feeding
2= Elliot et al. (1971), Co added to diet
3= Sutton and Elliot (1972), Co added to diet
4= Hedrich et al. (1973), Co added to diet
5= Rickard et al. (1975), Co added to diet

TABLE 1.3 Effect of the proportion of roughage in the diet on the levels of cobalamin and total vitamin B12 production for different cobalt intakes, plus the efficiency of cobalamin absorption.

Diet (% roughage)	Cbl production (nmol/d)	Total vitamin B12 production (nmol/d)	Cbl absorption efficiency (%)	Ref.
94	34-81	-	5	1
94	303-539	-	5	
100	1624-1653	2587-2599	2-20	2
0	338-612	1684-2421	0-16	
100	882	3402		
70	786	2807	1-35	3
40	445	2224		
70	27.3	643	0	
70	742	3649	15.2	4
70	1146	5133	19.4	
70	790	3726	-	
70	1690	4344	-	5
39	831	4437	-	
70	-	-	9.5	
70	-	-	37.9	6
70	-	-	22.0	
70	-	-	24.6	

0 represents a negative figure, i.e. a net secretion of Cbl into the small intestine.

References: 1= Smith and Marston (1970)
2= Elliot et al. (1971)
3= Sutton and Elliot (1972)
4= Hedrich et al. (1973)
5= Rickard et al. (1975)
6= Rickard and Elliot (1978)

have been studied by many workers, using a variety of diets. Production has been found to lie within the range 303-1690 nmol/d for Cbl and 1684-5133 nmol/d for total vitamin B12 from Co-adequate diets; on Co-deficient feeds Cbl production was only 27.3-81.2 nmol/d, while a single total vitamin B12 value of 643 nmol/d has been reported (Tables 1.2, 1.3). Mean values for the ruminal production of some analogues, compiled from samples using different diets, have been published. These were: (2-methyladenyl) cobamide - 964 nmol/d, cobinamide - 842 nmol/d, (adenine) cobamide - 241 nmol/d for diets having a Co content from 1.02-15.3 nmol/d, compared to a Cbl production of 1280 nmol/d (Bigger et al., 1976).

Vitamin B12 production changes rapidly in response to changes in Co intake. Tressol and Lamand (1979) found that total vitamin B12 levels in rumen contents responded within 24 h to addition or subtraction of Co from the diet. The response of Cbl may take longer to reach an equilibrium. After the withdrawal of a Co supplement from a deficient diet minimum values were not attained for 8 d (Smith and Marston, 1970a). Gawthorne (1970b), with the addition of Co to "Co-deficient rumen contents" incubated in vitro, found an increase in the levels of Cbl and (2-methyladenine) cobamide within 2 h. Other analogues appeared in the culture after 5 h, but constituted less than 6 % of the total vitamin B12.

The majority of workers have found the proportion of Cbl in total vitamin B12 produced to decrease with increased Co

intakes (Hine and Dawbarn, 1954; Gawthorne, 1970a; Smith and Marston, 1970a); however, Hedrich et al. (1973) found the reverse. The conversion efficiencies for incorporation of Co into Cbl are shown in Table 1.2. For sheep fed a roughage diet the proportion of Cbl in the total vitamin B12 produced was found to increase from 35-63 % as the Co content decreased from 5.77-0.679 µmol/kg diet; the analogue, (2-methyladenyl) cobamide, decreased from 49-36 % and (guanyl) cobamide formed 10-15 % at intakes greater than 1.70 µmol/kg, but was not detectable at lower levels (Gawthorne, 1970a).

Dietary composition Early work indicated that factors other than Co influenced ruminal total vitamin B12 production (Pearson et al., 1953; Hine and Dawbarn, 1954; Dawbarn et al., 1952; Hayes et al., 1966).

Further investigations have suggested that the presence of carbohydrate concentrates in the diet may decrease the proportion of Cbl in total vitamin B12 produced (Dryden and Hartman, 1971; Sutton and Elliot, 1972; Walker and Elliot, 1972; Rickard et al., 1975). Hedrich et al. (1973) attempted to quantify the effects of Co intake, percentage roughage in the diet and digestible dry matter intake (DDMI) upon daily duodenal Cbl flow (ruminal production). These factors accounted for 57 % of the total variation in production and predicted a decreasing proportion of Cbl produced when the percentage of concentrate in the diet increased (see Table 1.3). Using an in vitro system the

presence of nitrogen bases was shown to stimulate the synthesis of the corresponding cobamides at the expense of other cobamides (Gawthorne, 1970b). A similar qualitative response was found, in vivo, with the addition of 5,6 dimethylbenzimidazole to a 70 % roughage diet (Rickard et al., 1975).

High levels of molybdenum added to cattle rations (400 mg Co/kg diet) depressed vitamin B12 synthesis, but could be relieved by addition of Co (Davis et al., 1956). Pfander et al. (1966) suggested that high manganese intakes might interfere with Co utilisation by the host or its rumen microflora. Propionate added to the rumen of sheep and goats depressed total vitamin B12 activity of the contents (Roussev et al., 1975). This may be due to propionic-producing bacteria being among the major known ruminal producers of the vitamin, the inhibitory effect of VFA on microbial development and/or the stimulatory effect of propionic acid on the turnover of rumen contents. It has been suggested that a lower Co level is needed to maintain animal health on legumes than on grass forages (Pfander, 1966, cited by Looney et al., 1976). Such effects may be the reason for the differences in Co intestinal absorption (which includes Co as vitamin B12) when supplied by either legumes or ryegrass of comparable Co content (Grace, 1975).

It has been proposed that a lack of sufficient oxygen in the rumen might restrict Cbl synthesis (Menke, 1966, cited

by Walker, 1970). This could well be related to the finding of Horig and Renz (1979) that *in vitro* production of the cobalamin base 5,6-dimethylbenzimidazole, by *Propionibacterium freudenreichii*, was sensitive to variation in the oxygen concentration.

The majority of dietary studies have been undertaken using diets with a Co content greater than 1.87 µmol/kg DM, the recommended A.R.C. (1980) minimum. However, it is at low Co intakes that Co becomes nutritionally important.

Food intake Smith and Marston (1970a) found that Cbl production was limited by low levels of food intake, with or without adequate Co. The proportion of Cbl in total vitamin B12 produced increased from 19-38 % in response to a 40 % increase in DDMI and concomitant higher Co intake (Sutton and Elliot, 1972). However, total vitamin B12 production was not affected by the level of intake. Hedrich *et al*. (1973) using multiple regression analysis on an abundance of data suggested that the animal's DDMI had a negligible effect on Cbl production.

Cbl and analogues Of the 9 forms of the vitamin isolated from rumen contents, Cbl and (2-methyladenyl) cobamide predominate (Kon and Porter, 1954; Gawthorne, 1969). *In vitro* studies on pure rumen bacterial isolates have substantiated these findings (Dryden *et al*., 1962; National Institute for Research in Dairying, 1964).

Vitamin distribution within the rumen Smith and Marston (1970a) found that approximately 10 % of the Cbl and total vitamin B12 was freely detached from fibrous solid. Of that in the solid phase, 95 % was presumed to be associated with bacteria. The distribution of total vitamin B12 activity was shown by Dryden and Hartman (1971) to be uneven throughout the reticulo-rumen, but this effect was removed when expressed on a DM basis. Both bacteria and protozoa may be considered predominately solid phase organisms, but are capable of associating with the liquid phase (Hungate, 1966; Weller and Pilgrim, 1974). The liquid phase of rumen contents flows from the rumen at a greater rate than does the solid phase and both are influenced by the chemical composition, physical form and level of intake of the diet (Weller et al., 1962; McDonald et al., 1977) and consequently the flow of Cbl and analogues is affected.

Rumen microbial synthesis of cobalamin and analogues

There appears to be no information available on the production and/or requirements of Cbl and analogues by rumen protozoa; although Cbl, and upto 7 vitamin B12 analogues, have been found in rumen bacteria and rumen protozoa (Kon, 1955). The ingestion of bacteria by protozoa might provide sufficient vitamin for their needs. Rumen contents from Co-deficient animals were shown to have lower bacterial numbers and a loss of certain bacterial forms when compared to healthy animals (Gall et al., 1949).

More recently, Dryden and Hartman (1971) found a significant positive relationship between the total vitamin B12 rumen concentration and the number of viable bacteria. Such studies suggest a metabolic role for Co in bacteria.

Dryden et al. (1962) investigated the production of Cbl and analogues by rumen bacteria in vitro. Of 48 strains tested only 8 produced appreciable amounts. Selenomonas ruminantium and Peptostreptococcus elsdenii, common rumen microorganisms, were the most prolific producers, both of Cbl and analogues. Workers at Reading (National Institute for Research in Dairying, 1964) found that 47 out of 350 rumen bacterial isolates produced vitamin B12 and it was usually present in at least 2 forms, but that Cbl was not always produced. The range of species which synthesised the vitamin conflicted to some extent with the work of Dryden et al. (1962), but included Butyvibrio fibrosolvens and Bacteroides ruminicola which are amongst the most abundant rumen microbes. The characterisation of these species as significant contributors to ruminal vitamin production is in accord with the observed depletions of some bacteria in the rumen contents of Co-deficient sheep reported by Gall et al. (1949). When such losses occur the continuation of rumen fermentation is assured by the proliferation of other rumen microbes.

TABLE 1.4 The metabolic role of cobamides as coenzymes, with the organisms in which they are known to occur. "(methyl)cobamide" compiled from Poston and Stadtman (1975), "(5'-deoxy-5'-adenosyl)cobamide" compiled from Babior (1975).

Enzyme	Pathway	Source
(methyl)cobamide;		
N5-methyltetrahydrofolate: homocysteine methyltransferase	methionine synthesis	bacteria and mammals
unknown	methane synthesis	methanogenic bacteria
unknown	acetate synthesis	Clostridium sp.
(5'-deoxy-5'-adenosyl)cobamide;		
glutamate mutase	glutamic acid fermentation	Clostridium sp.
L-methylmalonyl CoA mutase	propionate metabolism	Propionibacteria sp. and mammals
⍺-methyleneglutarate mutase	nicotinic acid fermentation	Clostridium sp.
diol dehydrase	glycol metabolism	Aerobacter sp.
glycerol dehydrase	glycerol dehydrase	Aerobacter sp.
ethanolamine ammonia-lyase	vicinal amino alcohol deamination	Clostridium sp.
aminomutase	lysine fermentation	Clostridium sp.
ribonucleotide reductase	deoxyribonucleotide formation	Lactobacillus sp.

Metabolic role of corrinoids in microorganisms

That rumen microbes are capable of producing copious amounts of Cbl and analogues suggests a metabolic requirement for these compounds. Scott and Dehority (1965) demonstrated *in vitro* that a strain of *Ruminococcus flavefaciens* Cla had a growth requirement for Cbl that could be fulfilled by casein hydrolysate or methionine. This requirement is likely to relate to the coenzyme functions of the corrinoids (Table 1.4), and the action of the casein hydrolysate and methionine suggests an involvement in folic acid metabolism (p 49). While corrinoids other than Cbl can act coenzymically (Table 1.4), they all require either a methyl (Poston and Stadtman, 1975) or a 5'-deoxy-5'-adenosyl or closely related compound (Babior, 1975) in the Co β position (Fig. 1.1). Cbl is efficacious in all microbes known to require an exogenous supply of corrinoids. Many Cbl-requiring prokaryotes and simple eukaryotes can synthesise the entire Cbl molecule *de novo*, but some microbes and all higher eukaryotes require that it be supplied (Sennett *et al.*, 1981). Studies on the uptake of different forms of the vitamin by prokaryotes are few; most work has utilised *Escherichia coli*.

Those microbes requiring an exogenous supply of corrinoids may have absorption inhibited by competition between different forms of the vitamin for the membrane binding sites. Competitive inhibition of growth in

Poteriochromonas malhamensis (algae), Lactobacillus leichmannii (bacteria) and E.coli (bacteria), which all utilise Cbl, occurred with Cbl analogues (Ford, 1958, 1959; White et al., 1973; Kamikubo and Hayashi, 1979).

For those organisms capable of utilising analogues for growth it is not known whether these compounds are intrinsically active or undergo conversion to Cbl within the cell. Bacterial conversion of (2-methyladenine) cobamide to Cbl has been shown to occur (Ohlenroth and Friedman, 1968). Smith and Marston (1970a) found that CN-Cbl administered into the rumen was degraded to cobinamide; but Dawbarn et al. (1957a) found the level of cobinamide in rumen contents to be low, suggesting that bound Cbl may not be so readily degraded. Brandt et al. (1977) showed the formation of a nonadsorbable form of the vitamin from orally administered Cbl in the human small intestine; this was considered to be due to conversion by small-bowel bacterial overgrowth. Cobinamide added to in vitro rumen contents was found to be altered to a form utilisable by E.coli (Gawthorne, 1970a). At low levels of an antagonistic analogue, promotion of growth may occur by analogue saturation of extracellular binders (Ford, 1958). This is caused by the unavailability of the different vitamin forms if bound (Ternberg and Eakin, 1949; Smith, 1965; Grasbeck and Salonen, 1976).

L. leichmannii has been shown to store up to 9000-fold its nutritional requirement for Cbl (Kashket et al., 1962).

TABLE 1.5 Characteristics of some known human cobalamin binding proteins. Compiled from Grasbeck and Salonen (1976), Stenman, (1976) and Jacob et al. (1980).

	Structure	Mol. wt.	Source	Ligand
IF	glycoprotein	55,000	stomach parietal cells	Cbl
Cobalophilin *	glycoprotein	63,000	salivary gland	Cbl + analogues
Cobalophilin	glycoprotein	----	stomach mucosa	Cbl + analogues
Cobalophilin	glycoprotein	----	gall bladder	Cbl + analogues
Cobalophilin (TCI)	glycoprotein	56,000	plasma	Cbl + analogues
Cobalophilin (TCIII)	glycoprotein	----	plasma	Cbl + analogues
Transcobalamin (TCII)	protein	38,000	plasma	Cbl + analogues
Transcobalamin (TCO)	protein	----	plasma	Cbl + analogues

* R-protein was a term introduced to describe a non-IF Cbl binding protein in human gastric juice that moved more rapidly than IF on electrophoresis. Subsequently, immunologically identical proteins have been found in many body fluids. They have been found to bind more analogues than TCII. Cobalophilins differ in their carbohydrate, but not their protein, content. The use of the generic term cobalophilin is now considered more appropiate.

If a comparable situation exists in rumen microbes, then substantial amounts of the vitamin will be released in abomasal degeneration of microbial matter.

Intestinal transport and absorption of cobalamin and analogues

Cbl in the free form has been shown to be capable of permeating the rumen wall (Rerat et al., 1958a; 1958b). However, in natural materials the vitamin occurs largely in the bound state (Coates and Ford, 1955; Smith, 1965; Smith and Marston, 1970a) and absorption occurs at sites further along the alimentary tract, in the small intestine (Smith and Marston, 1970a). Little work has been undertaken on intestinal transport and absorption in ruminants, therefore it is pertinent to outline the understanding of monogastric absorption, despite the fact that the vitamin is present as a result of dietary intake, not gastrointestinal microbial production, and is predominately Cbl (Farquharson and Adams, 1976).

Gastric proteolytic enzymes release the vitamin from food protein linkages (Ellenbogen, 1975). The free molecule is then susceptible to binding by physiological proteins in the stomach millieu; these proteins (cobalophilins) are characterised in Table 1.5. At the low pH of the stomach both Cbl and analogues will preferentially bind to cobalophilin, secreted by the salivary glands and stomach mucosa, rather than intrinsic factor (IF), to form a stable complex. The affinities of Cbl for human salivary

cobalophilin are 50- and 3-fold higher than those for human IF at pH 2 and 8 respectively (Allen et al., 1978a).

In the duodenum, Cbl and cobalophilins are secreted in bile juice (Allen, 1975); thus recycling of the vitamin does occur. Pancreatic proteolytic digestion degrades cobalophilin, whether free or bound, so allowing the formation of IF-Cbl complexes (Allen et al., 1978a; 1978b; Parmentier et al., 1979; Marcoullis et al., 1980). The IF-Cbl complex is very stable and its integrity is maintained until absorption occurs at specific ileal microvilli receptor sites. Most other nutrients are absorbed in the jejenal region of the small intestine, rather than the ileal. IF from one species may facilitate Cbl absorption in another, e.g. hog stomach preparations relieve p.a. in humans.

Much less is understood about the passage of the vitamin through the ruminant gastrointestinal tract. The site of sheep IF production has been identified as the abomasal parietal cells (McKay and McLeay, 1981). Estimates of IF output vary from 10,500 to 23,500 international units/day (McKay and McLeay, 1981); sufficient to bind 8.12-17.7 nmol Cbl. 8.12 nmol Cbl/day being the requirement for sheep as determined by Smith and Marston (1970a). IF activity has also been demonstrated in cow rumen mucosa homogenates (Hippe and Schwartz, 1971). No studies appear to have been undertaken on the presence of gastrointestinal cobalophilins in the ruminant. However, serum

cobalophilins have been revealed in bovine serum (Polak et al., 1979), suggesting that similar binders might occur in salivary and biliary secretions.

Digesta moving into the acidic conditions of the abomasum will contain Cbl and analogues, most probably bound to microbial material and/or cobalophilins. With the death and degradation of viable bacteria, levels of the chemically bound vitamin should be increased substantially. Upon passage into the duodenum it might now be expected that degradation of free and bound cobalophilins by pancreatic proteases will ensure that only the stable IF-Cbl complex survives. It has been shown in vitro, at pH 7.0, that IF and cobalophilin are capable of sequestering Cbl from both viable and dead bacteria (Giannella et al., 1972). In contrast, Welkos et al. (1981) found that IF could not protect Cbl against binding by anaerobic gram negative bacteria isolated from rat intestine and maintained at pH 7. This competition may contribute significantly to the distribution of the vitamin during the ruminal digestive process. Marston (1970) thought that liberation of bacterial Cbl and the formation of IF-Cbl complexes would be inefficient, but further work (Smith and Marston, 1970a) suggested that release might be substantial and that the release of Cbl is largely paralleled by the release of analogue.

If these events occur then Cbl should be available for absorption in the small intestine. While little

information is available on the mechanism of ruminant absorption a number of estimates of absorption efficiency have been made (Table 1.3). At the present time the importance of IF in ruminant absorption is unknown. Elliot et al. (1971) did not discount the influence of diffusion in Cbl absorption. Passive absorption by diffusion is believed to occur in man when intestinal levels of the vitamin are high (Ellenbogen, 1975). Multiple regression analysis of data from sheep fed a 70 % roughage diet allowed Hedrich et al. (1973) to account for 57 % of the variation in apparent absorption as duodenal Cbl flow, ileal digesta flow and the percentage of the total vitamin B12 that was Cbl.

Faecal vitamin levels tend to reflect ruminal production in most instances (Hale et al., 1950; Dawbarn and Hine, 1955), but the contribution from microbial synthesis in the large intestine (Kercher and Smith, 1956) may significantly influence levels when diets are low in Co (Dawbarn and Hine, 1955). Such synthesis is, however, of no benefit to the host because this vitamin is not absorbed in the large intestine (Kercher and Smith, 1956). However, Jones and Anthony (1970) have attempted to derive a relationship between faecal total vitamin B12 and dietary Co, in order to determine the dietary Co status of ruminants in the field.

The site of Cbl absorption in ruminants is the small intestine; whether it occurs only in the ileal region, as

in man, is not known. In man, ileal absorption is known to be dependent upon calcium, a near neutral pH and have a receptor site, specific for the IF-Cbl complex, located in the microvillus membrane (Hall, 1979). Apart from the ability of the receptor to bind the IF-Cbl complex there is little indication of whether IF is split from Cbl on the receptor or within the ileal cell. Cbl is eventually bound to the serum transport protein transcobalamin (TC) II (Table 1.5) prior to release into the bloodstream (Hall, 1979).

In man, different forms of Cbl, e.g. methyl, cyano, are absorbed equally well, but analogues are poorly absorbed (Ellenbogen, 1975). Analogues of Cbl have been found in the blood of both ruminants (Dawbarn et al., 1957b; Sutton and Elliot, 1972) and man (Kolhouse et al., 1978) suggesting that they are absorbed, either with or without the IF mechanism. Rickard and Elliot (1978) found that added cobinamide was absorbed to the extent of 7-22 % of the labelled dose and that this was not immediately excreted. Analogue absorption may well be a passive process, dependent upon levels of analogues or proportions of analogues: Cbl.

Vascular and storage forms of Cbl and functions

The 3 human serum binders capable of Cbl and analogue binding (the transcobalamins; TCI, II and III) have

recently been shown to be present in bovine serum and able to bind cobinamide (Polak et al., 1979). Both the total binding capacity and the unbound binding capacity, 1.26 and 0.649 nmol Cbl/l serum respectively, were slightly lower for the bovine than for man. TCII was more saturated in the ruminant (50 %) than in man (40 %); but the reverse was true for TCI (94 and 96 % respectively) and TCII (55 and 70 % respectively). Linnell et al. (1979) found TCII, but not TCI or III, in sheep plasma and TCII bound 82 % of the plasma Cbl. In man, Cbl is predominately bound to TCI (England et al., 1976). Another vitamin B12 binding protein, TC0, has been found in blood, but it only bound a small percentage of the plasma Cbl and its function is unknown (Jacob et al., 1980). Of the human serum binders only TCII is known to be essential for Cbl absorption and transport (Jacob et al., 1980). TCI and TCIII are considered to be related to non-IF binders present in gastrointestinal secretions and are more appropriately termed cobalophilins (Stenman, 1976; Table 1.5). However, their physiological role is not understood, even though TCI may bind most of the human plasma Cbl (Jacob et al., 1980).

Human TCII is synthesised by many body cells; hepatocytes, fibroblasts, and macrophages have all been implicated in its production and enterocytic production seems probable (Jacob et al., 1980). TCII rapidly disperses Cbl and is then subject to enzymic degradation prior to excretion (Hall, 1979). Because of the faster turnover of the

TCII-Cbl complex it is this binder that is important in human serum Cbl transport. Cobalophilins (TCI, TCIII and gastrointestinal binders) are produced by many tissues, as reflected in their location throughout the body (Table 1.5). A small fraction of the cobalophilin-Cbl complex is secreted in bile. The understanding of the role of Cbl binders is based on limited knowledge and has been extensively reviewed by Jacob et al. (1980). Due to the varied distribution of Cbl between serum binders from different genera (Linnell et al., 1979), it would be unwise to draw too many parallels between man and ruminants with regard to the role of these serum binders (England and Linnell, 1979).

Serum and liver cobalamin concentrations in ruminants

Levels of Cbl in ruminant serum and body tissue reflect the animal's dietary history. An understanding of the relationship between serum and liver values and the clinical symptoms of Co deficiency has enabled reference ranges to be established and for blood Cbl levels to be used as diagnostic tests (Andrews et al., 1960; Marston, 1970; Sutherland, 1980).

Somers and Gawthorne (1967) found the diurnal and day to day variation in the concentration of total vitamin B12 in sheep serum to be large for animals with a dietary Co intake in excess of 1.70 μmol Co/kg diet, but small for animals on a lower intake. Tressol and Lamond (1979) found that a change in roughage diet from Co-adequate (3.40

µmol/kg DM) to Co-deficient (0.679 µmol/kg DM) caused total vitamin B12 serum values to fall after 1 week and achieve a minimum after 3 months. The addition of Co to the rumen of the animal fed the deficient diet increased the serum total vitamin B12 value within 12 h and a maximim was attained after 3 weeks. These changes in plasma Cbl will be affected by the Cbl status of the liver, as this organ has a limited capacity for storage. A given Co supplement will increase the plasma Cbl concentration more in animals of high initial liver Cbl status than in those of low initial status. Thus in most circumstances, serum and liver levels show poor correlation (Walker and Elliot, 1972; Millar and Penrose, 1980; Sutherland, 1980). While emphasis has been placed on liver values as indicators of the risk of deficiency (Millar and Penrose, 1980; Clark et al., 1981), the importance and interrelationship between serum and liver values in the definition of ovine Cbl status has recently been discussed (Sutherland, 1980).

In dairy cows fed a restricted roughage-high concentrate diet, Elliot (1980) found the liver Cbl to be lowered in early lactation even though serum total vitamin B12 levels were higher. He suggested that this might be due to the preferential ruminal synthesis of analogues and interference by these analogues during Cbl absorption and/or transport. Such phenomena could obviously contribute to the poor relationship between serum and liver values.

Despite their various shortcomings, serum and liver Cbl levels for sheep, as measured by the L.leichmannii assay, are recommended for diagnostic purposes (COSAC, 1982).

State	Serum (pmol/l)	Liver (nmol/kg wet wt)
deficient	<148	<73.8
borderline	148-296	73.8-140
adequate	>296	>140

The level for onset of Co deficiency in cattle is the same as for sheep, but cattle are generally regarded as being less susceptible to the condition (COSAC, 1982). The importance of the Cbl analytical technique is highlighted by the higher recommended serum levels when using radioisotope techniques (Sutherland, 1980; Clark et al., 1981). Reasons for these differences will be discussed later in this chapter (p 55).

That low plasma levels may be adequate in unstressed animals was recently noted by MacPherson (1981). Calves on a Co-deficient diet gained weight at the same rate as Co supplemented animals when housed, but not when exposed to the more stressful conditions outdoors. Diagnostic serum levels for cattle tend to be lower than those for sheep (COSAC, 1982). This may be due to a lack of relevant information on bovines, but is more probably due to genuine physiological differences, as suggested by Elliot (1980).

However, liver values are similar in both species. Findlay (1972) found adult sheep to have higher serum levels than young animals.

Cobalamin as a mammalian coenzyme

Cbl functions as a mammalian coenzyme in 2 forms, as listed in Table 1.4. Both 5'-deoxy-5'-adenosyl- and methyl-Cbl have Co in the +1 oxidation state, are photolabile and easily and quickly oxidised to the coenzymically inactive +3 state; the state in CN- and hydroxo-Cbl (Herbert and Das, 1976). Methyl-Cbl accounts for 50 to 90 % of the total circulating Cbl in man, but only 13 % in the sheep (Linnell et al., 1979). At the cell membrane, receptors facilitate Cbl absorption and movement within the cell (Jacob et al., 1980).

Cbl taken up by rabbit or rat liver cells has been shown to become associated principally with 1 of 2 proteins. The associated protein, which is present in the cytosol, is 5-methyltetrahydrofolate-homocysteine transmethylase (EC 2.1.1.13) and it is associated with Cbl in the hydroxo- and methyl- forms; the other protein in the mitochondria is L-methylmalonyl-CoA mutase (EC 5.4.99.2) in association with the 5'-deoxy-5'-adenosyl coenzyme (Kolhouse and Allen, 1977b; Mellman et al., 1977). Hydroxo-Cbl may be the form delivered by TCII to the cell membrane and thus acts as a precursor or intermediate for both coenzyme forms (Mahoney and Rosenberg, 1975; Matthews and Linnell, 1979). In humans the liver contains more Cbl than any other organ,

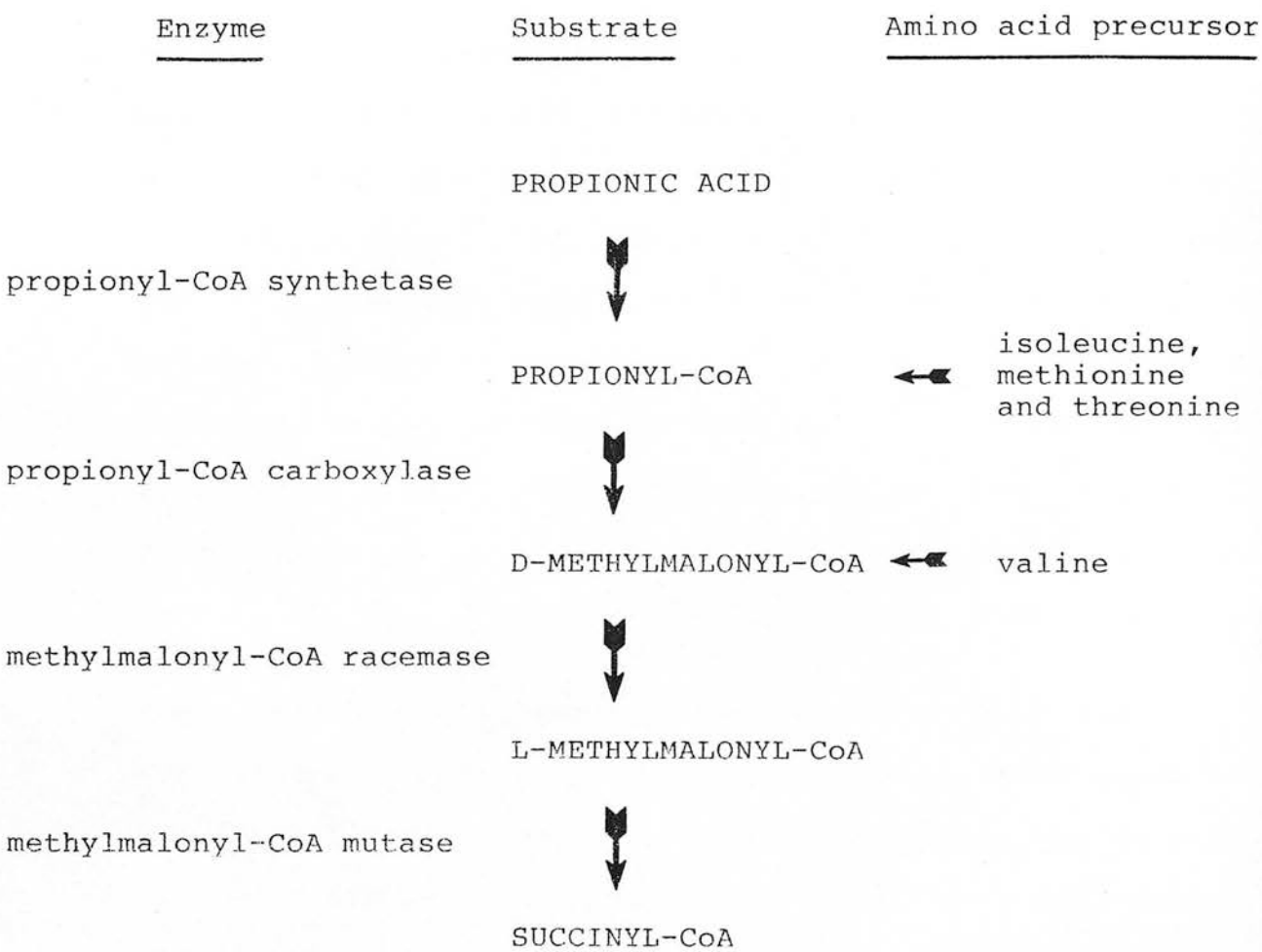


FIG. 1.3 Initial steps in propionate metabolism.

with 5-deoxy-5'-adenosyl-Cbl as the dominant form. This suggests that the high Cbl levels found in such organs may perform functions other than storage (Linnell, 1975).

Early studies with sheep showed that the total vitamin B12 levels of body tissues differed between organs and that there was a decrease in the levels in all organs of the deficient animal (Kercher and Smith, 1956). More recently the importance of the liver and kidneys in Cbl distribution has been confirmed (Smith and Marston, 1970a) and high levels in the rumen mucosa have been noted (Elliot and Hughes, 1976).

The ultimate fate of Cbl in the animal is unknown since no catobolic pathway has been revealed, though small amounts are lost daily in the urine and faeces (Linnell, 1975).

5'-deoxy-5'-adenosyl-cobalamin

The reversible conversion of L-methylmalony-CoA (MM-CoA) to succinyl-CoA requires 5'-deoxy-5'-adenosyl-Cbl as a coenzyme for L-methylmalonyl-CoA mutase (EC 5.4.99.2). This isomerisation occurs in the mitochondria and is an essential step in propionate catabolism (Marston et al., 1961). The amino acids isoleucine, threonine, methionine and valine can also be catabolised via this pathway, before entry into the tricarboxylic acid cycle (Lehninger, 1971; Fig. 1.3).

Cbl is quantitatively important to the ruminant because propionate is the major precursor of glucose in the fed

ruminant (Elliot, 1980) and Rickard and Elliot (1982) found differences in propionate metabolism to be correlated with the normal range of liver vitamin B12 values (148-812 nmol/kg liver), but these differences were not reflected in feed intake or the growth rate. However, Corse and Elliot (1970) did not find serum vitamin B12 levels to correlate with propionate clearance.

In non-ruminants, propionyl-CoA is derived from odd-chain fatty acids and catabolism of some amino acids and is not quantitatively significant (Beck, 1975). Propionate in the portal vein is removed by the liver, metabolised to succinyl-CoA (Fig. 1.3) which then enters the tricarboxylic acid cycle, ultimately to be converted into glucose (McDonald et al., 1977). It is therefore not surprising to find high levels of Cbl contained in the liver (Underwood, 1977). The form of Cbl in the ruminant's liver has not been identified, but would be expected to be predominately the 5'-deoxy-5'-adenosyl-Cbl coenzyme. Inter-species differences amoung ruminants, with regard to the efficiency of MM-CoA metabolism, have been postulated on the basis of differences in the branched chain fatty acid composition of body fat (Duncan and Garton, 1978). While the majority of propionate is metabolised in the liver, other tissues, e.g. the mammary gland, may use small amounts (Elliot, 1980).

Gluconeogenesis from amino acids can occur when the latter are present in excess of the animal's requirements or when

body tissues are utilised to maintain essential body processes, e.g. starvation (McDonald et al., 1977). In such instances, the carbon skeletons of isoleucine, threonine, methionine, and valine may be degraded via propionyl-CoA and MM-CoA and enter the tricarboxylic acid cycle (Elliot, 1980).

The metabolic consequences of a Cbl deficiency in the ruminant were shown to include an inadequate clearance of absorbed propionate and elevated blood propionate levels (Somers, 1969; Smith and Marston, 1971). Higher blood propionate levels were suggested as the cause of inappetence (Marston et al., 1972), a clinical sign of Co deficiency. The biochemical lesion caused by a 5'-deoxy-5'-adenosyl-Cbl deficiency should produce an increase in levels of methylmalonic acid (MMA), as in the absence of the coenzyme, MM-CoA is hydrolysed to MMA (Cardinale et al., 1969). However, this is only evident in the severely depleted animal because the potential accumulation of MMA is offset by a progressive decrease in the amount of propionate presented for metabolism, as feed intake declines (Gawthorne, 1968; Smith et al., 1969).

Work to derive and validate urinary (Andrews et al., 1970; Andrews and Hogan, 1972; Millar and Lorentz, 1979; Clark et al., 1981) and serum (Judson et al., 1981) MMA levels as an aid to diagnosis of Cbl deficiency has been undertaken. However, the enhanced urinary excretion of MMA for sheep fed barley diets (Lough and Calder, 1976) may be sufficient

to confound such a diagnosis. Levels in excess of 170 μmol MMA/l urine are considered to be indicative of a deficient condition. Furthermore, Elliot et al. (1979) drew attention to the possiblity of an overestimation of MMA levels with some of the analytical techniques used.

Biochemical abnormalities affecting lipid synthesis Severe Cbl deficiency can be accompanied by a neuropathy that becomes irrevesible if not treated (Beck, 1975). In 1970, the in vitro observation that MM-CoA accumulation in rat liver inhibited fatty acid synthesis (Cardinale et al., 1970) led to investigations of lipid metabolism and the formation of abnormal myelin in Cbl deficiency. As the majority of work has been undertaken on humans it is worth emphasising the degree of difference between the 2 species. In man, the precursors of MM-CoA are mainly amino acids and the urinary excretion of MMA is measured in milligrams per day, whereas in sheep the main precursor is propionic acid and because of the levels of ruminal production and subsequent absorption urinary excretion of MMA may reach several grams per day (Fell, 1981).

Mammalian fatty acid synthesis ensures a supply of saturated and unsaturated even-numbered fatty acids, but synthesis is dependent upon malonyl-CoA as the precursor (McDonald et al., 1977). Branched-chain (BCFA) and odd-numbered (ONFA) fatty acids normally occur in tissue in trace amounts only (Lehninger, 1971; Garton, 1975); but abnormal diets, such as those based largely on barley, or

rare congenital genetic defects are known to increase levels (Garton et al., 1972; Kishimoto et al., 1973). These long chain fatty acids are incorporated into acylglycerols for tissue storage and also have an important function in membrane lipids (Lehninger, 1971). In vitro work with sheep adipose tissue preparations showed that MM-CoA was incorporated into branched-chain fatty acids in the presence of malonyl-CoA (Scaife et al., 1978). Whereas propionyl-CoA acts as the direct precursor of ONFA (Garton et al., 1972). MM-CoA may serve as an alternative to malonyl-CoA in fatty acid synthesis if the L-MM-CoA mutase system is overloaded or if the level of coenzyme is reduced. Such an overloading was demonstrated by Duncan et al. (1974) in barley-fed lambs in which subcutaneous tissue had a greater proportion of propionate-derived fatty acids (BCFA and ONFA) than perinepheric tissue. The result was attributed to the inability of the liver to store or utilise sufficient coenzymic Cbl for the L-MMA-CoA mutase enzyme. Sheep fed barley were found to excrete excessive amounts of ethylmalmonic acid in addition to MMA and it was suggested that the former might also be used in fatty acid synthesis (Lough and Calder, 1976).

The incorporaton of BCFA and ONFA into depot lipids has been regarded as the explanation for lack of firmness in the subcutaneous fat of barley-fed lambs (Garton et al., 1972; Duncan et al., 1974; Spillane and L'Estrange, 1977). Similiar consequences in terms of lipid synthesis might be

expected when ruminants are deficient in Cbl. However, Duncan et al. (1981) found that lambs from Co-depleted ewes had elevated levels of ONFA in hepatic neutral- and phospho-lipids, as well as in adipose triacylglycerols, but that the levels of BCFA were normal. Such a response was considered to be due to the use of greater amounts of maternal propionate rather than maternal MMA in fatty acid synthesis.

Species differences in BCFA and ONFA were apparent when sheep and goats on cereal rich diets were found to have enhanced levels of BCFA and ONFA in adipose tissue, but cattle and red deer did not (Duncan and Garton, 1978; Wahle et al., 1979).

The importance of such compounds in the development of neurological disorders is indicated by reports of the deranged synthesis of fatty acids and associated effects on membrane structure (Frenkel, 1973; Frenkel et al., 1973; Frenkel et al., 1976).

Inhibition of normal fatty acid synthesis by alteration of propionate metabolism has been proposed for the low milk fat syndrome of cows fed a high-grain low-fibre diet (Frobish and Davis, 1977). However, so far there has been little evidence to substantiate this theory (Croom et al., 1981).

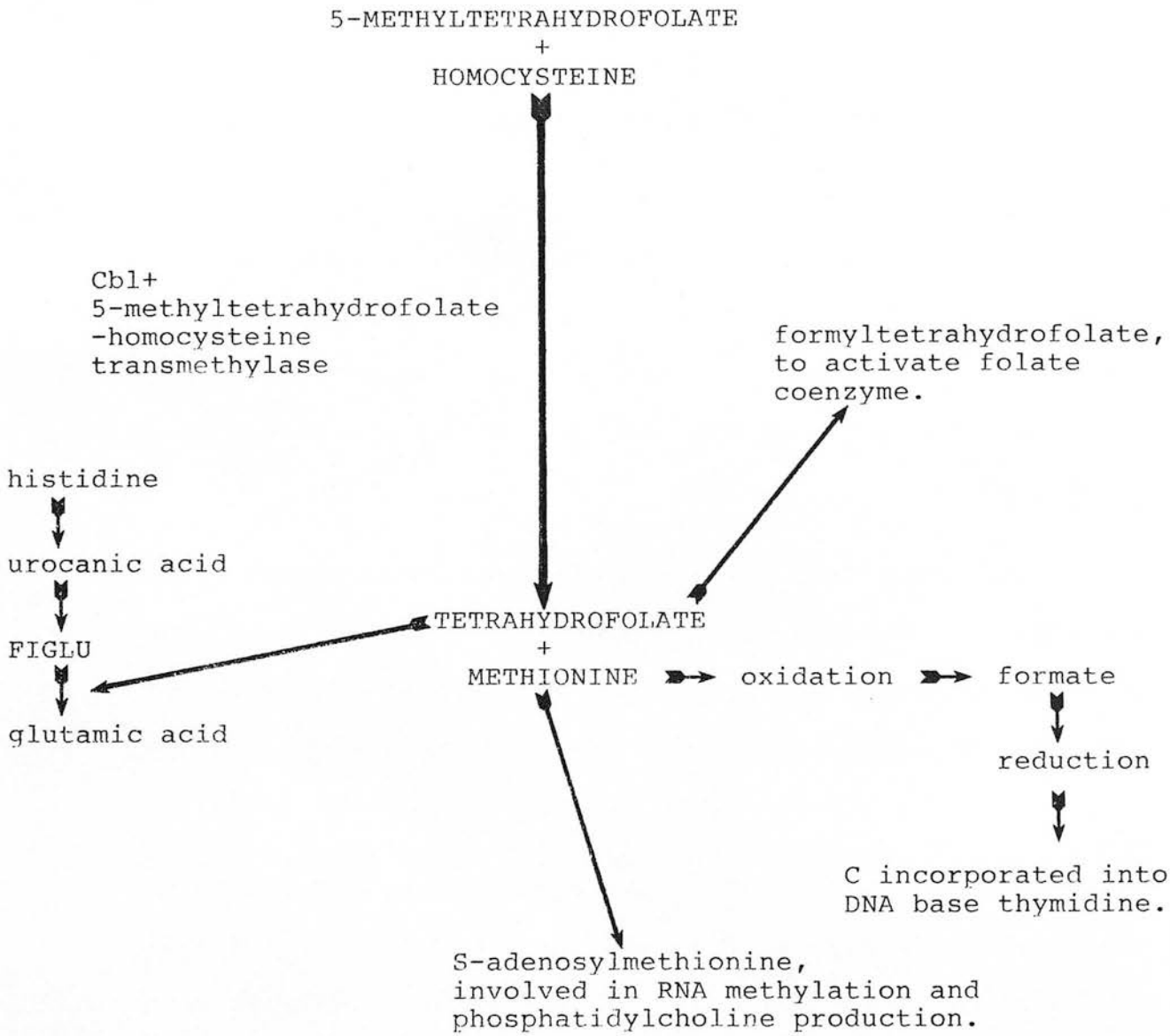


FIG. 1.4 Methionine synthesis and folate interactions.

Methylcobalamin

The second important function of Cbl is as the methyl-Cbl form, which acts as a coenzyme for 5-methyltetrahydrofolate-homocysteine transmethylase (EC 2.1.1.13), in conjunction with the cofactors S-adenosylmethionine and reduced flavin adenine dinucleotide ($FADH_2$), in the methylation of homocysteine to methionine (Fig. 1.4). The reaction has 2 important facets; first it generates methionine, required for the production of S-adenosylmethionine and formate; secondly tetrahydrofolate, the functional form of folate, is produced (Chanarin, 1981). Folate metabolism is deranged in severe Cbl deficiency (Herbert and Das, 1976). Folates (pteroylglutamic acid) are only biochemically active in the reduced state, i.e. 4 hydrogenations of the pteridine ring and the incorporation of a carbon adduct. Intracellular folate is predominately in the coenzymic state, which requires more than one glutamic acid residue to function, i.e. folate polyglutamates (Chanarin, 1980). Polyglutamates constitute 75 to 99 % of the total folates in sheep's liver (Osbourne-White and Smith, 1973). Formate is required for the production of formyl folates, the normal substrate for polyglutamate synthesis, from tetrahydrofolate (Chanarin et al., 1980). In contrast, Gawthorne and Smith (1973), using an in vitro sheep's liver cystol preparation, found that 5-formyltetrahydrofolate, 5-methyltetrahydrofolate and tetrahydofolate produced folate polyglutamates.

Lack of tetrahydrofolate in Cbl deficiency will block the folate-dependent metabolism of formiminoglutamic acid (FIGLU) to glutamic acid (Herbert and Zalusky, 1962; Fig. 1.4). Urinary levels of FIGLU are elevated in Co-deficient sheep and this is considered a more sensitive indicator of the deficient state than MMA (Gawthorne, 1968). Russel et al. (1975) suggested that the presence of FIGLU in urine is indicative of a Co-deficient state and that this parameter reflected the growth rate more accurately than serum vitamin B12 using the L.leichmannii assay. However, little work has been directed towards the establishment of diagnostic levels. Further evidence of the link between Cbl and folate metabolism is provided by reports that Co-deficient sheep have low levels of certain liver folates (Dawbarn et al., 1958; Smith and Osbourne-White, 1973; Smith et al., 1974). It has also been found that liver methyltetrahydrofolate, along with all fully reduced folate, was depleted in Co-deficient sheep and this was reversed by either Cbl or methionine injections (Smith et al., 1974). It is noteworthy that the administration of either compound prevented or provided remission of "fatty liver" in these animals. However, the amounts of methionine required for normal lipid metabolism in the deficient sheep were inadequate for maintenance of liver folates. This failure was ascribed to an impairment in the transport of folates into liver in the Co-deficient animal (Gawthorne and Smith, 1974).

Folates are also involved in de novo purine synthesis, thus exerting an influence on nucleic acid production, a fact that has been utilised in the deoxyuridine (dU) suppression test for megaloblastosis caused by Cbl and/or folate deficiency (Herbert et al., 1973).

Biochemical abnormalities causing anaemia In man Cbl deficiency is directly associated with the methyl-Cbl coenzyme and the development of a unique megaloblastic anaemia (England and Linnell, 1979). The anaemia is considered to be due to an impairment in the synthesis of the DNA base thymidine, which normally occurs by a carbon addition to deoxyuridylate. Impaired methionine synthesis gives rise to lower levels of formate, the principle carbon donor (Fig. 1.4), this is the "de novo", as opposed to the "salvage" pathway (Herbert and Das, 1976). In other animals studied the de novo pathway is relatively unimportant (Chanarin, 1981), which may account for the lack of megaloblatosis. Anaemia in Co-deficient ruminants has not been investigated thoroughly, although it has long been recognised (Underwood and Filmer, 1935). In sheep it is normochromic, normocytic anaemia (Smith et al., 1950; Gawthorne et al., 1966; Fell, 1981), although Marston (1952) found it to be macrocytic, and it does not appear until the deficiency is well established, i.e. after loss of appetite. Haematological analysis has been suggested as being more reliable in diagnosis of the Cbl-deficient state than serum Cbl values, in both man (England and Linnell,

1979) and sheep (Sheriff and Habel, 1976). England and Linnell (1979) also suggested that the different haematological responses of man and other animals to Cbl deficiency was due to differences in the distribution and role of serum Cbl binders.

Biochemical abnormalities affecting neurology Methionine is a source of S-adenosylmethionine, a major methyl group donor (Lehninger, 1971). This compound is required for methylation of some RNA bases and of phosphatidylethanolamine (cephalin) to form phosphatidylcholine (lecithin) (Lehninger, 1971). These last mentioned are phosphoglycerides important in blood lipid transport and are also incorporated into nervous tissue and the brain. A defect in phosphoglyceride generation might be responsible for the "fatty liver" condition, through immobilisation of depot lipids. Phosphoglycerides are also the major component of most animal cell membranes. The liver content of S-adenosylmethionine was found to be lowered in Cbl-deficient sheep (Gawthorne and Smith, 1974), and this may explain the decrease in the conversion of phosphatidylethanolamine to phosphatidylcholine found in Co-deficient ewes (Lough et al., 1982). Cycloleucine, an inhibitor of S-adenosylmethionine formation, has been shown to produce a neuropathy in mice with myelin sheath degeneration (Jacobsen and Gandy, 1973) and an impaired methylation of myelin basic proteins (Craig and Jacobsen, 1980).

Histopathology of cobalt deficiency

The histopathological effects of ruminant Co deficiency have recently been discussed by Fell (1981). He endorsed a previous report on the possible connection between ovine white liver disease and Co deficiency (Sutherland et al., 1979; Mitchell et al., 1982). Liver degeneration in Co-deficient sheep was noted by Macpherson et al. (1976), during a study on the possible involvement of Co in the aetiology of cerebrocortical necrosis. In Co-deficient sheep, neurons of the cerebral cortex and brain stem underwent atrophy and degeneration; while white matter in the spinal cord became vacuolated due to demyelination and axonal loss. The muscular atrophy associated with severe Co deficiency was general throughout the skeletal muscle and the specific fibre type atrophy indicated a process of denervation or disuse (Fell, 1981).

Quantitative requirements of ruminants for cobalamin and their fulfillment

Marston (1970) and Smith and Marston (1970a) have estimated the minimum total requirements for sheep at approximately 8.12 nmol Cbl/d. Those estimates are in agreement with those of Hedrich et al. (1973). Sensitivity to Co-deficiency differs among ruminants. Under grazing conditions lambs are more sensitive, then mature sheep, calves and mature cattle in that order (Andrews, 1956).

For ruminants in which Co-deficiency is likely to occur, the element can be supplied as a regular oral dose or in the form of a Co bullet placed into the rumen. Oral dosing at 119 µmol is required weekly (Lee, 1950), while a Co bullet ahould last at least 3 months. However, bullets are not well retained by cattle, nor by certain sheep breeds, e.g. Romney (Andrews, 1971). They are also subject to coating with calcium phosphate in the rumen, which prevents Co release. In both cases investigations should be continued to ensure that remission of the deficiency is occurring.

Oral and parenteral applications of Cbl, while being effective, are not recommended because of the cost and inconvenience.

Effects of cobalt upon animal reproduction

Apart from the effects of Co, via Cbl production, already mentioned various other effects have been ascribed to an inadequate Co intake. These have been assiduously compiled by Young (1979) and cover many biochemical aspects. More pertinent, perhaps, are the few reports of the effects of Co deficiency upon reproductive performance. These suggest that an inadequate Co intake can effect reproductive performance in both sheep (Duncan et al., 1981) and cattle (Hidiroglou, 1979; Musewe and Gombe, 1980).

Methods of vitamin B12 analysis

Elucidation of the complexities of vitamin B12 biochemistry and physiology reflected the constant development of analytical methods for the vitamin. Preoccupation with the notion that p.a. was a unique human disease inhibited the search for assay methods involving growth in laboratory animals, until the isolation of Cbl in 1948. Then, methods of biological assay using both microbial and higher animals were developed. The evolution of these 2 forms of assay will be considered separately and problems that are common to both will be discussed later.

Animal growth assays

Although chicks and rats were used to assay a.p.f. before the isolation of Cbl it was after 1948 that the development of these methods expanded (Smith, 1965). There are 2 main difficulties associated with the use of higher animals.

1. Young animals usually carry considerable reserves of the vitamin, which are depleted only slowly on a Cbl-deficient diet. Therefore, breeding stock needed to be kept on a diet deficient in the vitamin so that their offspring were depleted at birth. Alternatively, it was found that the problem of depletion could be circumvented by increasing the animal's requirement for Cbl through the feeding of thyroid-active materials, e.g. iodinated casein or thyroxine (Ershoff, 1947).

2. Cbl is synthesised by the gut flora and may be absorbed from the gut: it is also excreted in the faeces so the test animal must be housed in such a way that it has no access to them. That coprophagy can increase the total vitamin B12 level of the stomach contents was shown by Morgan et al. (1961) using rats.

Chicks and rodents were the predominate higher organisms used for biological assay. Chick assays have been described by Nichol et al. (1949) and Coates et al. (1951b; 1956), while assays utilising rats or mice have been proposed by a number of groups (Bosshardt et al., 1949; Cutherbertson and Thornton, 1952; Frost et al., 1953).

Weight gains in response to unknown extracts were tested against pure Cbl, or standard extracts. Chick assays covered a range of 5.53-11.1 (Nichol et al., 1949) and 3.69-22.1 nmol vitamin B12/kg diet (Coates et al., 1951b) for assays of 14 d and 28 d duration respectively. The mice and rat assays were performed over similar periods (Bosshardt et al., 1949; Frost et al., 1953) with a standard range for the rat assay of 0-7.38 nmol vitamin B12/kg diet (Frost et al., 1953). Antibiotics in the diet were shown to influence growth with and without vitamin B12 (Coates et al., 1951c) and inclusion of penicillin and aureomycin in the diet interfered with the response in the rat assay (Frost et al., 1953). Assays employing chicks and rats remained popular until the mid-1950's.

Microbial growth assays

Many microorganisms require Cbl for growth and those that became established in assays will be considered individually.

Lactobacillus species In 1947 Shorb reported that liver extracts increased the growth of the bacillus Lactobacillus lactis Dorner (American Type Culture Collection, ATCC 8000). Following the isolation of Cbl, a microbial assay using turbidity of the culture as a measure of growth was devised (Shorb, 1948). The complex growth requirements of this organism and its inhibition by certain substances (Shorb and Briggs, 1948) made its routine use difficult and unpredictable, and it was soon superseded by Lactobacillus leichmannii (ATCC 7830, Hoffmann et al., 1948; 1949; ATCC 4797, Skeggs et al., 1950).

A complication with all the lactobacilli is that they respond to deoxyribonucleotides in addition to Cbl (Hoffmann et al., 1948). Some workers have attempted to apply a correction for this by destroying the Cbl using heat at an alkaline pH (Ross, 1950; Rosenthal and Sarett, 1952). Any residual response was presumed to represent the non-vitamin B12 interfering substances and the assay result was corrected accordingly. Coates and Ford (1955) were critical of this since they found that the Cbl content of liver and fish solubles was barely diminished by autoclaving at pH 11. A further complication is that some derivatives of vitamin B12 have been shown to interfere

with the uptake of Cbl in L.leichmannii (Kashket et al., 1962). However, its advantages are, that the assay incubation period is much shorter than that in animal assays and the useful range for the L.leichmannii (ATCC 7830) assay is lower at 0-18.5 pmol Cbl/l. Despite its shortcomings it is still routinely used in both medical and veterinary laboratories for serum analysis.

Escherichia coli Davis and Mingioli (1950) isolated several Cbl-requiring mutants of E.coli (ATCC 9637) and Harrison et al. (1951) used strain 113-3 (Davis) in a cup plate assay for the vitamin. This technique uses a solid agar plate seeded with the microorganism. Standards or unknowns are placed in wells cut into the agar. The constituents of the samples diffuse into the surrounding agar causing growth of the microorganism and a consequent increase in turbidity. The diameters of the "zones of exhibition" are assumed to be proportional to the concentration of vitamin B12. The range of this assay is 36.9-36,900 pmol Cbl/l sample. Burkholder (1951) increased the sensitivity using test tube cultures of E.coli and this is now the more common form of assay. Standards or unknowns are incorporated in a liquid medium to which is added the inoculum and microbial growth, measured as turbidity of the culture, is again proportional to the concentration of vitamin present. The range of the test tube assay is 29.5-177 pmol Cbl/l culture media. However, these 2

TABLE 1.6 Response of the different microbial assays to the major forms of vitamin B12 in the rumen (Smith, 1965).

Base of nucleotide	E. coli (plate)	E. coli (tube)	L. leichmannii (tube)	E. gracilis (tube)	P.malhamensis (tube)
5,6 dimethylbenzimidazole	100	100	100	100	100
Adenine	100	10	50	100	0
2-methyladenine	100	50	40	60	0
No nucleotide	100-250	20	0	0	0

N.B. All other compounds are in relation to Cbl (5,6 dimethylbenzimidazole) arbitarily measured at 100.

techniques respond differently to the various analogues of vitamin B12 (Fantes et al., 1956a) and can give different total vitamin B12 values for the same sample (Table 1.6). The significance of these values will be discussed later. A number of substances, notably methionine, are known to interfere with E.coli assays (Cutherbertson et al., 1951) and they are not widely used. However, the results of the assay using human serum compared well with those of E.gracilis z (Sourial, 1981) and it is used as a bioautographing agent after separation of the different forms of the vitamin (Gawthorne, 1969; Linnell et al., 1970).

Euglena gracilis Bacteria are not the only microorganisms to be exploited in vitamin B12 assays. Hutner and his colleagues (1949) found that the photosythetic alga Euglena gracilis var. bacillaris (Culture Centre of Algae and Protozoa, CCAP 1224/7a) had a Cbl requirement and this microorganism was subsequently employed by Robbins et al. (1950) in a bioassay. When utilised for the assay of Cbl in serum 2 inadequacies became apparent; excessive increases in pH during algal growth caused precipitation of the serum proteins and the long incubation period, 7-10 days, delayed results. These problems were overcome by the introduction of the z strain of E.gracilis (CCAP 1224/5z) and improvement of the medium (Hutner et al., 1956). Use of a buffered medium at pH 3.6 had a bacteriostatic effect and turbidity caused by the precipitation of serum proteins during incubation was minimised. The modified assay was completed in 3 to 5 days. Recognition of large inter-assay

variations (Nicholas and Pitney, 1958; Shinton, 1959) prompted Anderson to investigate optimisation of the assay conditions (1964). She included small volumes of serum in the standards, which affected growth, and standardised the incubation conditions. The range of the assay is 0-14.8 pmol Cbl/l and as little as 0.185 pmol Cbl/l can be detected (Hutner et al., 1956).

Poteriochromonas malhamensis Hutner et al. (1953) were also responsible for the elucidation of the growth requirements of Ochromonas malhamensis. This facultative photosynthetic alga has now been reclassified as Poteriochromonas malhamensis (CCAP 933/1a). Ford (1953) modified the medium of Hutner et al. in order to measure the Cbl levels in some crude extracts. He later showed that certain natural and synthetic analogues of vitamin B12 could inhibit growth, apparently by binding competitively (1958; 1959; Ford et al., 1955a). Similar conclusions were reached by Kamikubo and Hayashi (1979). Despite this, it is widely used as the reference method for Cbl since it is considered not to respond positively to analogues in plasma (Rickard et al., 1975) or rumen contents (Elliot et al., 1971). The range of this assay is 0-118 pmol Cbl/l.

Interference by antibiotics and other drugs

Antibiotics applied in human medicine have been found to interfere in some of the microbial assays. In 1967 Watts demonstrated the sensitivity, in vitro, of L.leichmannii to a number of antibiotics but claimed that dilution, as well as the heat and precipitation of protein involved in sample preparation, would remove the antibiotic in most instances.

Powell et al. (1969) showed that some antibiotics, e.g. ampicillin, were not destroyed prior to assaying and that their presence inhibited growth, while Boczarow (1961) found that serum samples from patients on penicillin inhibited the growth of L.leichmannii. However, L.leichmannii is not the only microorganism to be inhibited by drugs; Herbert et al. (1965) reported the inhibition of E.gracilis by chlorpromazine but this drug did not inhibit growth of L.leichmannii (Forshaw and Harwood, 1966; Powell et al., 1969). In practice, many drugs that might affect microbial assays are often diluted out because of the extreme sensitivity of most of the assays (Baker et al., 1981). Both E.gracilis and P.malhamensis will be unaffected by bacterial antibiotics. Veterinary use of antibiotics may also be the cause of interference in microbial assays, although this has received little attention.

Comparison between bioassays

With the development of so many bioassay methods comparison of the same biological material, using different techniques, became popular. Hoffmann et al. (1949) and Frost et al. (1953), using liver extracts found good agreement between the microbial assays and those using chicks and rats. As the range of materials under investigation expanded, so differences appeared and the nonspecificity of most assays began to be appreciated (Hoffmann et al., 1949; Pierce et al., 1949; Kaczka et al., 1950; Ford et al., 1951; Smith et al., 1951a; Coates et al., 1952b; Ford and Porter, 1952; Coates et al., 1953).

Assays of natural material, using chicks, tended to give results higher in value than those of microbial assay (Coates et al., 1951b). This was considered to be due to the a.p.f. However, the reverse was true when the gut contents and faeces of ruminants were assayed using chicks, E.coli and L.leichmannii (Coates et al., 1951a; 1953).

A crude distinction was made between those substances that could be converted to CN-Cbl by the action of cyanide and those that, although producing growth in a microorganism, could not (Ford et al., 1951; Coates et al., 1952a; ; Ford and Porter, 1952). These compounds could be separated by chromatography (Ford et al., 1951) and/or electrophoresis (Ford et al., 1953a; Brown et al., 1955) and determined by bioautography (Ford and Holdsworth, 1953; Ford and Porter, 1953; Dawbarn et al., 1957a).

Workers at Shinfield produced evidence that gut organisms were able to effect interconversions between different analogues of vitamin B12 (Coates et al., 1953; Ford and Porter, 1953). The biosynthesis of analogues allowed a large number to be tested for biological activity (Ford et al., 1955b; Funk and Nathan, 1958) and microbial assays have now been characterised by the form of vitamin B12 they will respond to (Table 1.6).

P.malhamensis is considered the most specific, responding only to Cbl, whilst E.coli is widely used in bioautography because it is the least discriminating. Generally, analogues with a purine as the base only show "full B12 activity" towards the least discriminating microorganisms,

such as E.coli; they show less activity towards other microorganisms and little or none towards P.malhamensis or higher animals (Smith, 1965). It has been stated that for any single sample the growth response to vitamin B12 would differ in the order, E.coli(plate)>= E.coli(tube) >=L.leichmannii >=E.gracilis >=P.malhamensis (Smith,1965). However, other authors have altered the order slightly (Ford, 1953; Cook and Ellis, 1968) and differences would be expected for samples differing in analogue content, e.g. yeast extract cf. plasma.

An important consequence of the different responses to analogues is that it is seldom possible when using bioassays to express with precision the potency of an analogue in relation to that of Cbl (Fantes et al., 1956b). Not only can the relative potency vary from one test organism to another but also between the form of the assays (Table 1.6). For preparations containing mixtures of active compounds quantitative values will only be achieved by prior, chromatographic or electrophoretic, separation and assaying against the appropriate standard. This process is lengthy and is not routinely employed. Instead, bioassay results are commonly measured against a Cbl standard and expressed as "vitamin B12 activity". For body fluids, in which most of the vitamin is present as Cbl, then L.leichmannii is commonly used and the results may be very informative; particularly if a laboratory has sufficient data to construct its own reference range. But for tissues and other natural materials results should be interpreted with great caution (Ford, 1952; Coates and Ford, 1955; Cook and Ellis, 1968).

The importance of sample preparation

In 1949 Ternberg and Eakin showed that Cbl combines with a non-dialysable component of normal gastric juice and that this bound vitamin was unavailable to L.leichmannii. That the vitamin occurs predominately in natural material as the bound form was shown by many workers (Ross, 1950; Rosenthal and Sarett, 1952; Ross, 1952; Coates et al., 1953; Gregory, 1954; Pitney et al., 1954). Liberation of the vitamin was shown to occur for most samples when they are heated, particularly when this was performed at an acid pH (Ross, 1950; Rosenthal and Sarett, 1952; Ross, 1952). A temperature of 100 °C maintained for 30 m gave maximum results (Ross, 1952): this can be achieved using a waterbath, but the alternative of an autoclave has been queried because of a lack of reproducibility that was considered to be the result of excessive autoclaving (Orrell and Caswell, 1972).

Values were shown to be further increased when a trace of cyanide was added to the sample before heating (Coates et al., 1951a; De Heus and De Man, 1951; Dawbarn and Hine, 1954; Spray, 1955; Matthews, 1962). The addition of cyanide is thought to perform 2 functions; it facilitates extraction of the vitamin (Wijmenga et al., 1950; Denton and Kellog, 1953), possibly by displacing protein or peptide groups linked to the vitamin (Coates and Ford, 1955) and it converts Cbl (and analogous compounds) to their more stable cyano forms (Tarr, 1951; Ford, 1953; Spray, 1955). In the cyano form the vitamin gives a greater growth response than hydroxo-Cbl when L.lactis or

L.leichmannii is used (Cooperman et al., 1951), a response which may be related to it binding less strongly than hydroxo-Cbl to the serum proteins (Skeggs et al., 1960). These effects were confirmed by Coates and Ford (1955) who questioned the validity of assays performed on samples prepared in the absence of cyanide. The presence of a reducing agent in the media was also believed to protect the vitamin from destruction during autoclaving (Hoffmann et al., 1949; Cooperman et al., 1951). Heating samples to 100 °C for 30 m in the presence of acid and cyanide will generally liberate the vitamin, but some samples, e.g milk, tissue, require prior enzymic digestion (Gregory et al., 1952; 1954; Green, 1980).

Precipitated proteins must be removed before assaying to prevent interference with turbidity measurements. The media normally used with E.gracilis was of sufficiently low pH to minimise protein precipitation and consequently samples could be assayed without prior heat treatment. This afforded the opportunity, by using treated and untreated samples, to determine quantitatively the importance of the binding proteins.

Radioisotopic assay

In 1960, Yalow and Berson described a radioimmunassay for insulin in body fluids that used the specificity of the antigen-antibody relationship. In the same year Ekins reported the use of a competitive protein binding (c.p.b.) method, with a naturally occurring binding protein in place of an antibody, for a thyroxine assay. The same theory is applicable to both and is generally termed saturation analysis (Ekins, 1974).

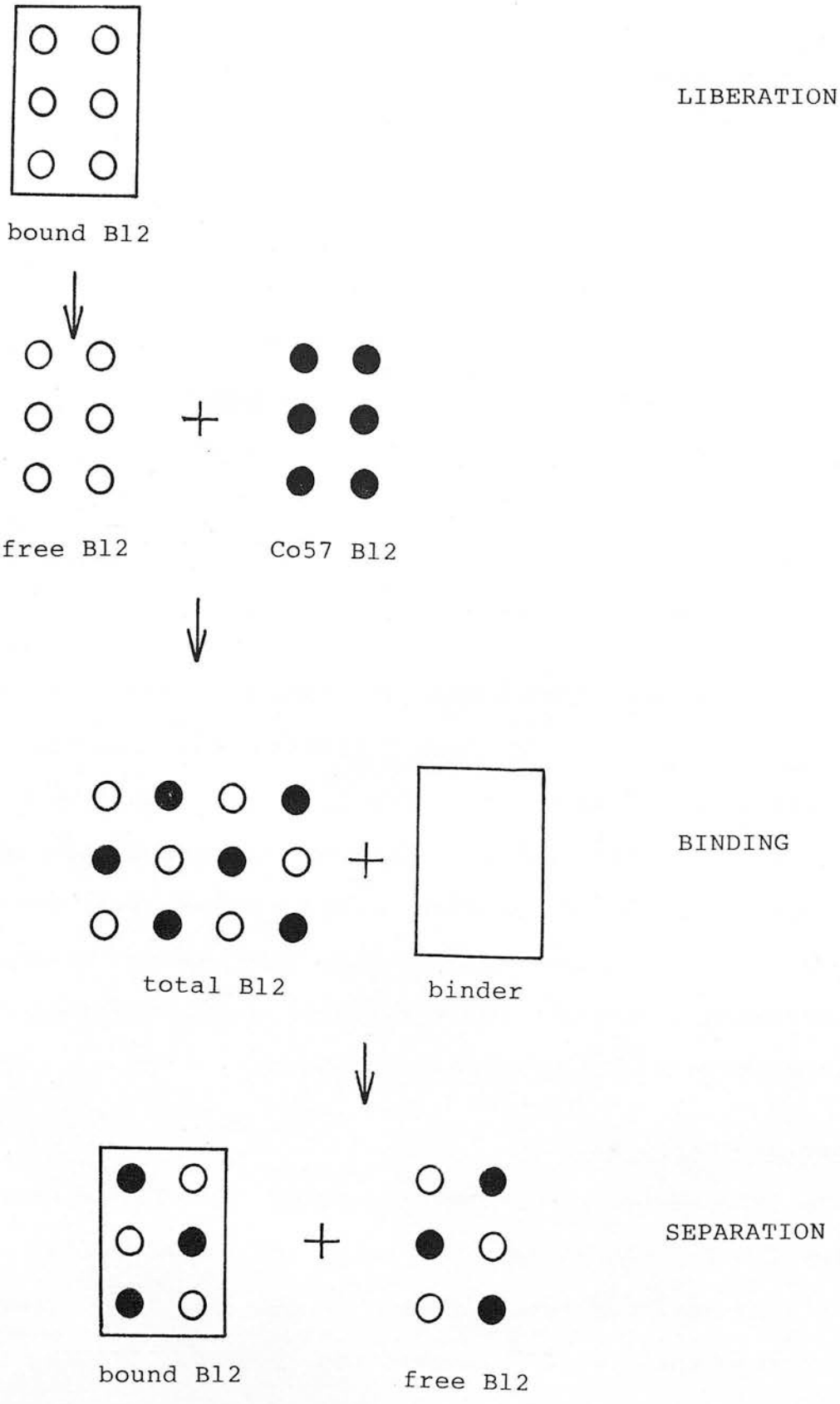


FIG. 1.5 Scheme of competitive protein binding radioisotopic assay for vitamin B12.

In both methods a specific receptor, antibody or binding protein, is saturated by the substance to be measured (ligand) and the bound: free ligand ratio is a function of the total amount of ligand present. An unknown amount of ligand may be quantified by comparing its distribution with those of standards. Radioactively labelled ligands can be used to measure the distribution between compartments. An unknown amount of labelled ligand is added to both standards and unknowns prior to binding and the partition of the tracer measured after binding and separation. It is assumed that the unlabelled ligand behaves identically to the labelled. This principle forms the basis of the c.p.b. method for vitamin B12 analysis (Fig. 1.5). The use of radioactivity is not a fundamental feature of saturation analysis. The label can be any material which can be measured, e.g. a fluorescing antibody (fluoroimmunoassay).

Saturation analysis of vitamin B12 has 3 critical steps.

Liberation - the release of endogenous vitamin from its binding protein in the biological sample.

Binding - addition of labelled Cbl to the extracted vitamin and exposure to a new binder.

Separation - the separation of free and bound forms of the vitamin.

The early development of these techniques was specifically for the analysis of human serum samples. A fact that has recently been shown to be important when other biological samples are considered.

After the relationship between Cbl and an IF preparation had been demonstrated (Herbert, 1959; Herbert et al., 1960) an assay was developed using IF as binder (Rothenberg, 1961). For separation the bound complex was precipitated using barium hydroxide and zinc sulphate. Other assays used serum as the binding agent with dialysis (Barakat and Ekins, 1961) or adsorption onto charcoal (Grossowicz et al., 1962) for separation. The use of charcoal was later shown to introduce errors (Gottlieb et al., 1965). All 3 techniques used heating of the serum sample, at an acid pH, for liberation and in 1965 Lau and co-workers improved its effectiveness. Liberation of the vitamin was achieved with protein denaturation rather than precipitation, by adding a stronger hydrochloric acid (0.25 N) to the serum. Hence, removal of coagulated protein was avoided. Serum binders would not be effective after this treatment (Herbert et al., 1960; Rothenberg, 1963) and Lau et al. (1965) followed Herbert et al. (1960; 1964) in using IF as the binder and they separated the free and bound vitamin with protein coated charcoal. Many modifications have since been made to this technique for each of the 3 steps.

Liberation Problems caused by coagulated proteins (Rothenburg, 1963; 1968) were intended to be removed by protein denaturation with a strong acid, but this was also found to interfere (Raven et al., 1966; 1968; 1969). Tibbling (1969) and Frenkel et al., (1970) therefore suggested that treatment of serum should be at a higher buffered pH, e.g. pH 4.0, but stressed the potential for residual or nonspecific binding of endogenous vitamin in all

techniques. Most techniques now utilise a buffer of approximately this value (Rothenburg, 1968; Jacobs and Zonday, 1969; Roos, 1970; Ceska and Lundkvist, 1971; Wide and Killander, 1971; Buchanan et al., 1977; Green, 1980; Reynoso et al., 1981). Some proprietary kits investigated by LeFebvre et al., (1980) used alkaline conditions for liberation, but in 1 instance this produced high nonspecific binding and gave falsely low results.

As with the microbial assay the addition of cyanide at liberation improved reproducibility (Hall, 1966; Raven et al., 1968; Jacobs and Zondag, 1969; Frenkel et al., 1970; Liu and Sullivan, 1972), but the optimum level of cyanide has not yet been resolved (Rothenburg, 1981). The importance of an exact temperature for liberation, i.e. autoclave or waterbath, has also been investigated by different workers (Raven et al., 1968; Orrell and Caswell, 1971).

Binding Radioassay techniques all require labelled material of high purity, as the radioactive ligand is added in more than trace amounts and methods that enable the purity and concentration to be checked have been described (Barlow and Sanderson, 1960; Frenkel et al., 1970).

Many workers have followed Lau et al. (1965) in using IF as the binding protein (Hillman et al., 1969; Jacobs and Zondag, 1969; Raven et al., 1969; Rubini, 1970; Ceska and Lundkvist, 1971; Wide and Killander, 1971; Liu and Sullivan, 1972; Puutula and Stenman, 1974; Gutcho and Mansbach, 1977; Reynoso et al., 1981). IF gained

popularity as a binder because of its availability and purported specificity, but it had several limitations. Rothenberg (1963; 1968) demonstrated that IF was unstable when used at low concentrations in the assay and that it had an affinity for glassware that might be responsible for the variable binding capacity that occurred with storage. Raven et al. (1969) were able to overcome such problems by increasing the concentration of their working solutions and by the use of polystyrene tubes. They also added vitamin B12-free serum to their standards since it increased the binding capacity of IF, a procedure adopted by other workers (Rothenburg, 1961; Hillman et al., 1969; Green et al., 1974; Begley and Hall, 1979).

Serum from different animals has also been used as a binder (Matthews et al., 1967; Britt et al., 1969; Frenkel et al., 1970; Newmark et al., 1973; Buchanan et al., 1977) in order to improve the specificity, accuracy and ease of assay. For chicken (Newmark et al., 1973) and toadfish (Kim et al., 1976) serum further addition of the sera to standards is unnecessary, both can be stored frozen with no change in binding capacity and chicken serum has a very high affinity for Cbl, such that precipitated proteins do not have to be removed prior to binding. Other binding proteins used are TCI (Rothenburg, 1968), TCII (Judson et al., 1982), saliva (Carmel and Coltman, 1969) and "dog stomach binders" (Hippe and Oleson, 1975).

The continued search for new binders is a reflection of the lack of understanding of those first used. The problems arose partly from pH-dependent changes in binding

capacity (Goldberg and Fudenburg, 1969; Rose and Chanarin, 1969; Hippe and Olesen, 1971; Shum et al., 1971; Newmark et al., 1973; Allen et al., 1978a; 1978b; Kolhouse et al., 1978). The range of maximum binding varies between binders; most attain a maximum at alkaline pH (ca. pH 9.0) with extreme pH causing a decrease in capacity. Kolhouse et al. (1978) found that human and hog cobalophilin retained their full Cbl binding capacity down to pH 4.0 and 2.0 respectively, but that both human and hog IF lost 10 % and 98 % of their capacity when at pH 4.0 and 2.0 respectively; values that were commonly used for the binding procedure. Binding capacity is also affected by ionic strength, particularly at low pH (Green, 1980), a phenomenon that may be related to the binding of the IF-Cbl complex to glass. Newmark and Patel (1971) found that IF binding to glass, which is maximal at low pH, could be overcome by "saturating" the glass with albumin.

In proprietary radioassay kits, that have IF as the stated binder, cobalophilin may provide upto 85 % of the binding capacity (Kolhouse et al., 1978). Such impurities might produce misleading results in view of the affinity of cobalophilin for vitamin B12 analogues (Kolhouse and Allen, 1977a). The problem may be overcome by the use of vitamin B12 analogues, such as dicyanide cobinamide, to saturate the nonspecific binding capacity of the contaminating cobalophilin (Begley and Trachtenberg, 1979; Cooper et al., 1979; Kubasik et al., 1980). The use of human serum, as a binder, is open to the same problems since it is capable of binding a number of vitamin B12 analogues, some with a

comparable affinity to that of Cbl (Bunge and Schilling, 1957; Gregory and Holdsworth, 1960; Meyer et al., 1963; Gottlieb et al., 1967). Work on the isolated serum binders TCI, TCII and granulocyte cobalophilin has shown that they are each capable of binding to certain analogues of vitamin B12 (Gottlieb et al., 1967; Hippe et al., 1971; Kolhouse and Allen, 1977a). When a sample contains a non-IF binder then competition for the binders between any vitamin B12 analogues present may produce erroneous results, regardless of the purity of the binder.

Separation The method of separation of free and bound forms of the vitamin can affect results, and it is itself influenced by the type of binder and form of Cbl used (Adams and McEwan, 1974). The coated charcoal method incorporated by Lau et al. (1965) is probably the most widely used, but incomplete removal of the free vitamin has been reported (LeFebvre et al., 1980). Other techniques have included ultrafiltration (Friedner et al., 1969), gel filtration (Mantoz et al., 1967), adsorption of the bound vitamin onto DEAE-cellulose (Frenkel et al., 1966; Tibbling, 1969) or Amberlite resin (Roos, 1970), and attachment of the vitamin binder to plastic test tubes (Rubini, 1970) or to Sephadex (Wide and Killander, 1971). Brombacher et al. (1972) emphasised that if coated charcoal was used then the time it remained in contact with the sample should be limited to avoid removing excessive amounts of the bound complex.

Separation of the free and bound vitamin is followed by radioactivity counting. Although either fraction could be

used most techniques count the bound form. Some assays (Lau et al., 1965; Kelly and Herbert, 1967; Raven et al., 1969) have based their calculations on the supposition that the assay procedure obeyed the principle of proportional (radioisotope) dilution (Rittenberg and Foster, 1940). This declares that for a doubling of ligand concentration then there would be a proportional decrease in percentage binding. Workers have found that this was not true for IF preparations (Raven et al., 1966; Rothenburg, 1968; Frenkel et al., 1970), but was for chicken serum (Newmark et al., 1973) and toadfish serum (Kim et al., 1976). When proportional dilution principles do not apply, the use of a standard curve is essential. There are many methods of expressing the standard curve (Ekins, 1974; Chard, 1978). In most instances transformations of the data are performed to achieve linearity and so simplify the calculations, the best known being the logit transformation (Rodbard and Lewald, 1970).

$$\text{logit } b = \text{natural log } \frac{b}{100-b}$$

Where b = proportion of the tracer bound expressed as a percentage of that bound in the zero standard.

The many variations in the development of this assay have all had the same goal; the rapid, accurate and specific analysis of vitamin B12 concentrations in serum. That they have occurred at all is a reflection of the complexity of vitamin B12 biochemistry and physiology. With the international acceptance of c.p.b. assays for vitamin analysis the United States National Committee for Clinical

Laboratory Standards (U.S. NCCLS) has now published guidelines for evaluating serum Cbl assays and recommends assessment of some 100 items (NCCLS, 1980).

Recently a radioimmunoassay for Cbl using a labelled methyl ester of the vitamin has been reported (Endres et al., 1978). Whether the development of such techniques will prove advantageous for routine clinical analysis has yet to be demonstrated.

Comparison between microbial and radioisotopic assays

Radioassay results for human serum are generally higher than those of microbial assay (Green et al., 1974; Buchanan et al., 1977; Mollin et al., 1980; Sourial, 1981), due possibly to a lack of specificity in some binders (Kolhouse et al., 1978; Kubasik et al., 1980). This explanation would require the presence of analogues in human serum; which have been reported (Kolhouse et al., 1978), although queried by England and Linnell (1980). The work of Sourial and Mollin (1979) suggests that analogues may be present in human serum in a form unavailable to even the least discriminating microbial assay (E.coli) and that their levels may parallel tissue stores. Sheppard and Ryrie (1980) found that levels of vitamin B12 analogues increased and those of Cbl decreased in human serum for patients suffering from folate deficiency, and that this was rectified by treatment wth folic acid.

That assays developed for the analysis of human serum might not always be applicable to other biological fluids was shown by Millar and Penrose (1980). Five out of 6 commercial radioassay kits were found to work with sheep sera and these correlated well with the L.leichmannii microbial assay, but generally gave slightly higher values. More recently, differences in nonspecific binding between ovine and bovine sera when analysed by commercial radioassay kits were reported (Wright et al., 1982). The use of biological fluids rich in different forms of vitamin B12, as in this study, necessitates a rigorous approach in the validation of methods of analysis for the vitamin.

OBJECTIVES

The development of the continuous culture rumen simulation technique (Rusitec) has allowed for experiments of long duration to be undertaken, with a minimum of sophisticated technology. This study was to investigate the feasibility of such a system for trace element work. The aims being to examine dietary and microbial factors influencing the incorporation of Co into vitamin B12 in the rumen. The advantage of such a study is that it can concentrate upon the synthesising rumen bacteria without host interaction. Knowledge thus gained might facilitate a new approach to the prediction and prevention of Co deficiency through the accurate assessment of the "Co potency" of the diet and subsequent optimisation of Cbl absorption.

CHAPTER 2

MATERIALS AND METHODS

Rumen simulation

Ruminants are characterised by the development of a pregastric, microbial, digestive system. Fermentation in the rumen by the symbiotic, microbial population digests complex plant carbohydrates. The products of this fermentation, in addition to the microbial matter itself, then become a source of nutrients for the host. Within the rumen a multitude of reactions and interactions occur and hence investigation is extremely difficult, often necessitating long-term studies in the living animal under controlled conditions. However, for preliminary work and/or routine assays such as the determination of feed digestibility, *in vitro* systems offer a large degree of control and economy. It was therefore decided to use an *in vitro* system in this investigation to study vitamin B12 production by rumen microbes.

A continuous culture method, in which the substrates are maintained and waste products removed over an extended period, was selected because this more closely approaches the situation *in vivo*, than short-term, batch culture methods. That divergence between *in vivo* and *in vitro* reactions might occur was recognised, but as Hungate (1966) concluded, "the value of the continuous fermentation model is not so much that it is exactly applicable but that it provides a model for reference." The degree of

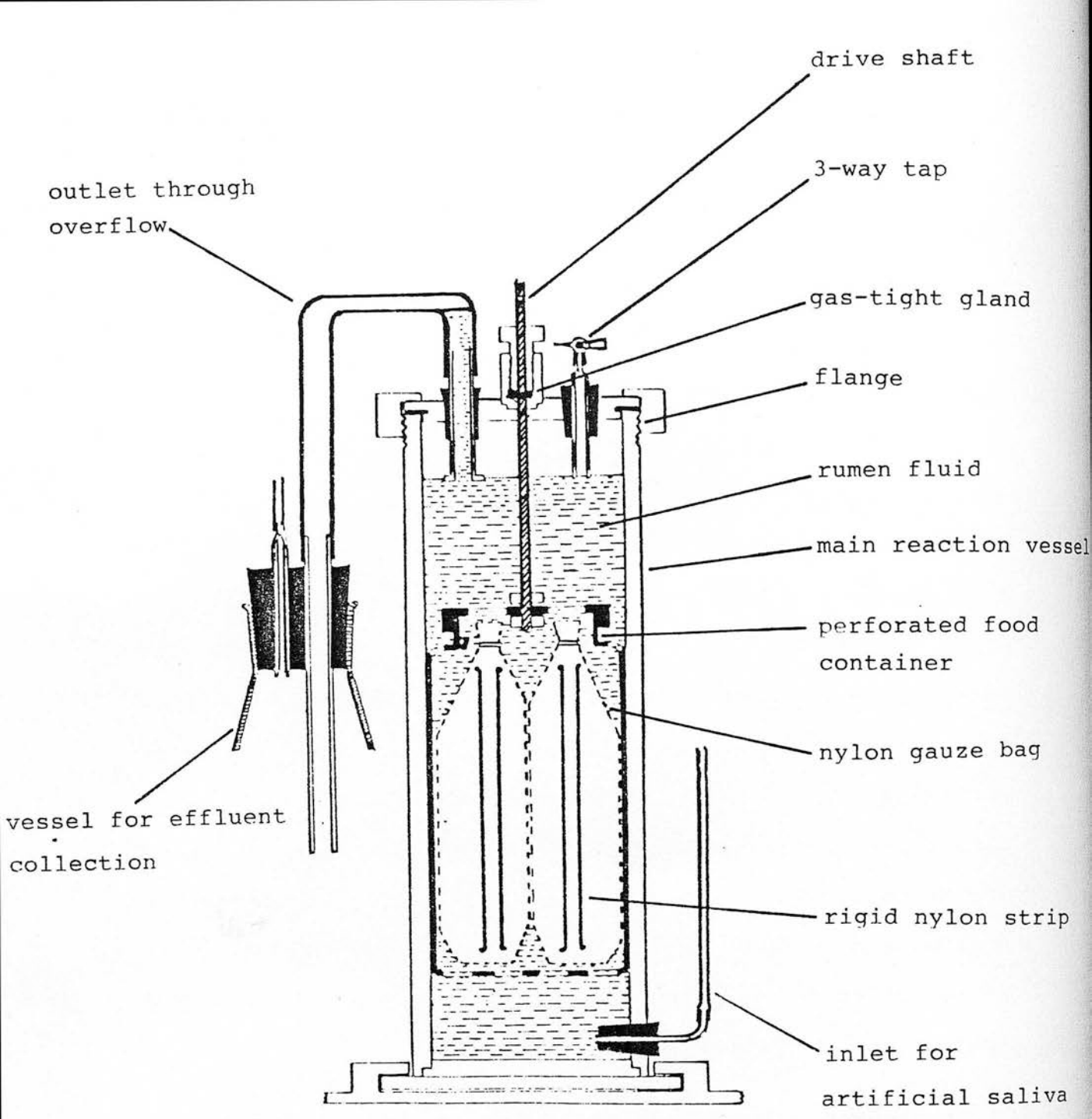


FIG. 2.1 Schematic diagram of a single unit of the long-term artificial rumen (Rusitec).

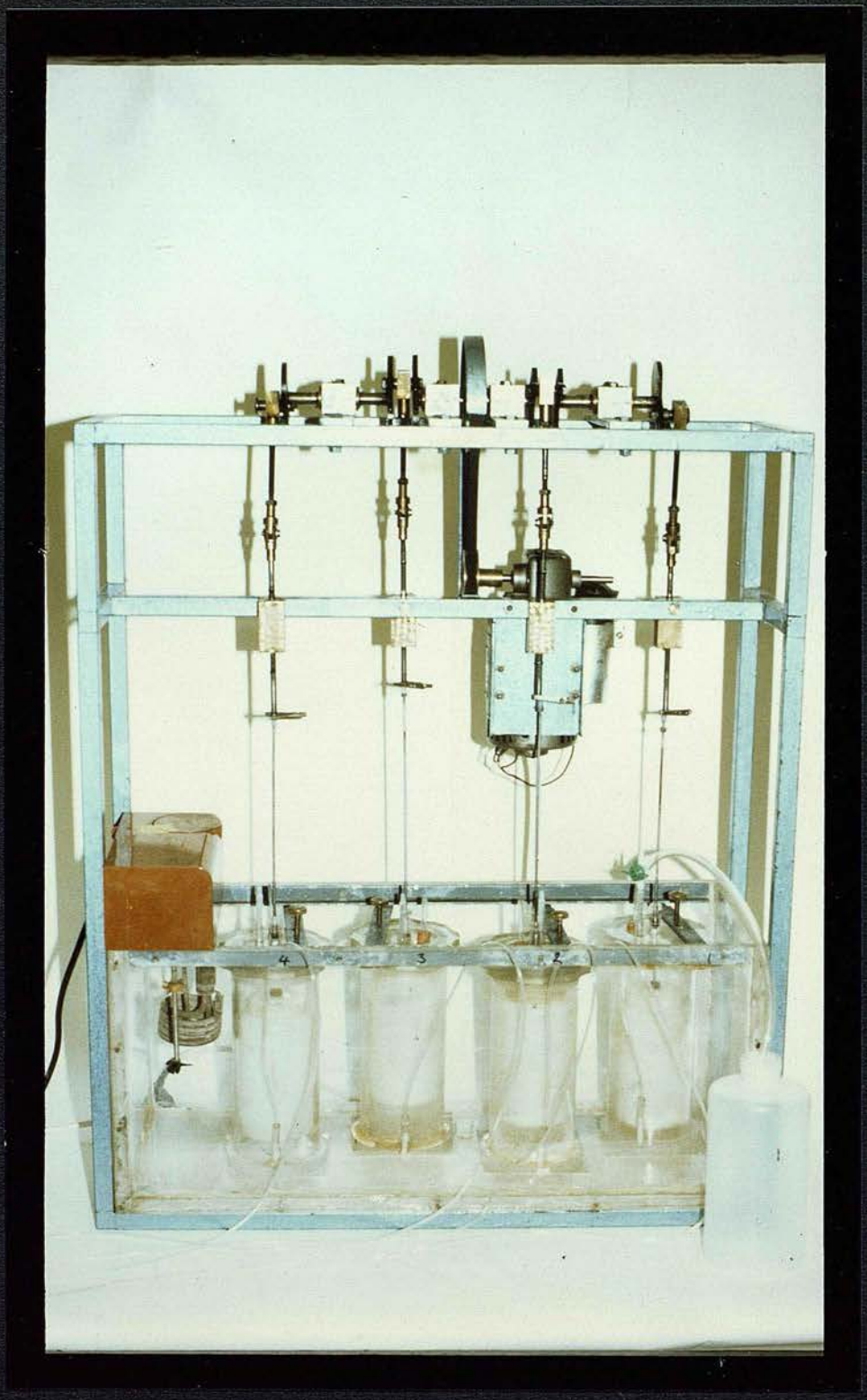


PLATE 2.1 The four-vessel Rusitec used in these experiments.

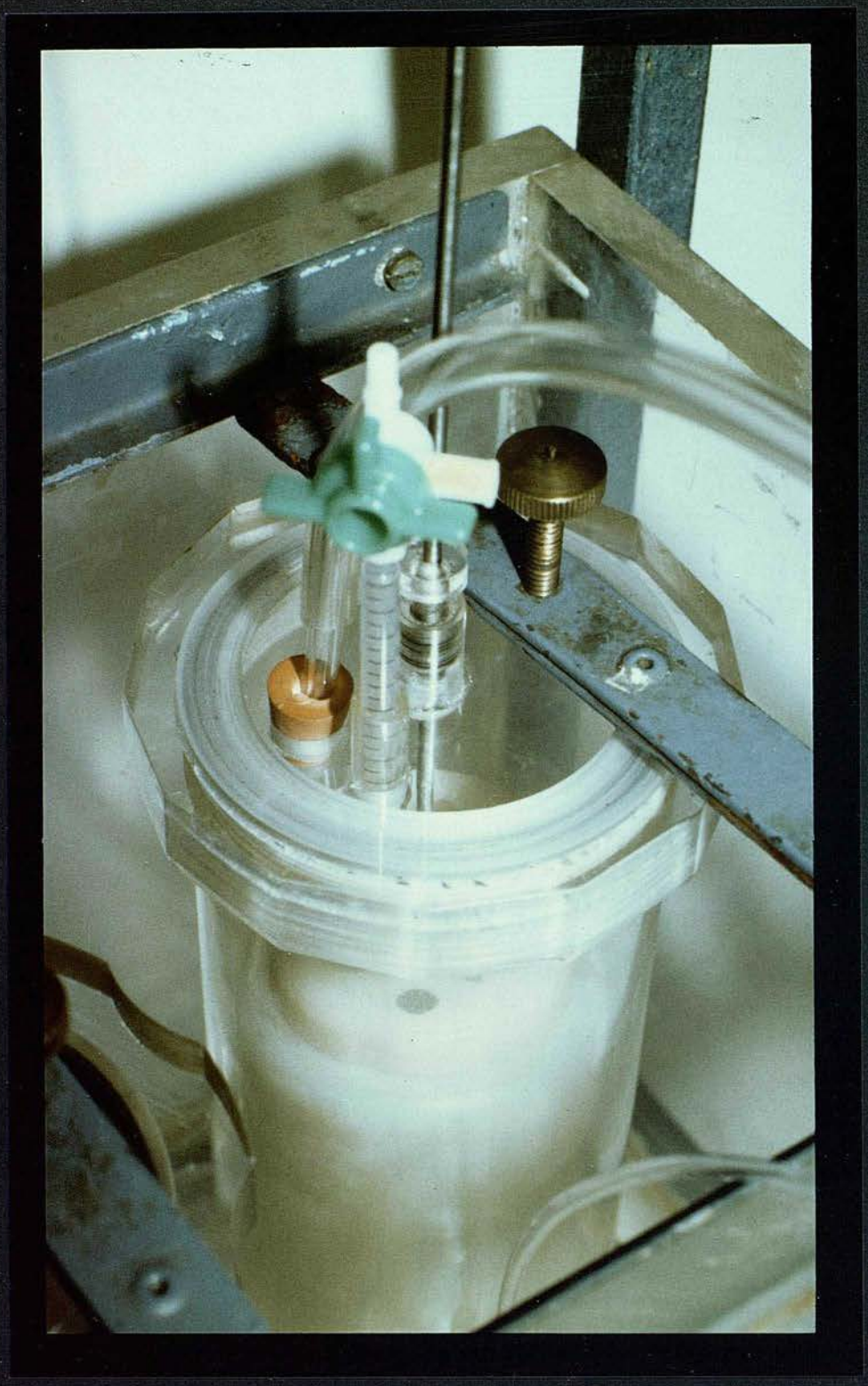


PLATE 2.2 A view, in situ, of the top of one vessel from Rusitec. Protruding from the top of the clamped vessel are the three-way tap and the effluent overflow.

sophistication for *in vitro* continuous culture work can be great (Ewart, 1974), but the method chosen for this study was the relatively simple rumen simulation technique (Rusitec), devised by Czerkawski and Breckenridge (1977); which has been shown to simulate volatile fatty acid (VFA) production on both concentrate and roughage rations (Czerkawski and Breckenridge, 1977).

The apparatus consists of 4 vessels constructed as uniformly as possible to ensure identical conditions. Each vessel has a total capacity of 1000 ml and consists of a perspex cylinder (200 x 80 mm i.d.) with an inlet on the base and sealed by a flat perspex cover provided with a screw flange (Fig. 2.1; Plates 2.1, 2.2). Two holes in the cover allow for overflow to a polythene collecting vessel and sampling via a 3-way tap (Southern Syringe Services Ltd., London). The overflow port protrudes below the cover so that, when sealed, gas collected above the vessel fluid gives sufficient pressure to assist the flow of effluent into the collecting vessel. The volume of the vessel to the overflow is approximately 800 ml. All 4 vessels are enclosed in a waterbath, in the base of which there were recesses for positioning of the vessels. The waterbath was maintained at a temperature of 39 ± 0.05 °C by means of a heater unit fitted with a stirrer (Grant Instruments Ltd., Cambridge).

In using Rusitec, daily rations of food were placed in bags (200 x 100 mm) made from fine mesh nylon (1 perforation /mm) used in wine making (The Boots Co. Ltd., Nottingham). Also included was a nylon stiffener to

TABLE 2.1 Composition of "artificial saliva" (McDougall, 1948)

Compound	Amount(g)
$NaHCO_3$	9.8
$Na_2HPO_4.12H_2O$	9.3
NaCl	0.47
KCl	0.57
$CaCl_2.2H_2O$	0.053
$MgCl_2.6H_2O$	0.13

All compounds were Analar reagents supplied by BDH Chemicals Ltd., Poole, Dorset and were made up to one litre with deionised, distilled water.

prevent collapse of the bag, which was finally closed using plastic ties (Plasti-tie, Synchemicals Ltd., London). The bag was placed in a wide-mouthed, polystyrene container with a screw cap of the same material; the container gave a tight, sliding fit in the reaction vessel. Fluid could pass through the container via holes drilled in the base and the screw cap and the container was moved vertically in the vessel by a stainless steel rod passing through a gas tight gland in the vessel cover and connected to the container's screw cap and to a crank actuated by a motor. 'O' rings were used around the perspex cover and within the gland to ensure a gas-tight seal. The motor speed was 14 r.p.m. (Klaxon Ltd., Birmingham) and geared to produce a vertical stroke of 30-40 mm at 8 cycles /m.

On the first day of an experiment, solid rumen contents (80 g) were placed in a nylon bag and one of these, together with a bag of the proposed ration, was placed inside the food container in each vessel. The cap was screwed on and the assembly lowered into the reaction vessel. To fill the reaction vessel, 500 ml strained rumen fluid, 200 ml artificial saliva (McDougall, 1948; Table 2.1) and 100 ml distilled, deionised water was used. Distilled, deionised water was used throughout this project unless otherwise stated. The perspex cover was tightened, the vessel located in the recessed base and connection to the crank was performed quickly using a crocodile clip. Movement of the reaction vessel was prevented by a metal clamp across the top of the vessel. Anaerobic conditions were ensured immediately by flushing each vessel for a few

minutes with nitrogen (B.O.C., London) via the 3-way tap. The tube was placed in the effluent collecting vessel and the motor started.

Fermentation gases displaced into the collecting vessel were allowed to escape. When samples of effluent were to be collected, 5 ml sodium cyanide (0.06 M) was placed in the collecting vessel to inhibit further fermentation and vitamin B12 production. Although the use of sodium cyanide may not have completely prevented anaerobic respiration the lower temperature of the collecting vessel and the aerobic conditions would also have subdued fermentation considerably. This was seen to be the case when analysis of samples taken throughout 24 h from cyanide-treated Rustic fluid showed no further production of Cbl in the effluent. Cyanide will not prevent the adsorption of Cbl or cyanide cobinamide in E.coli at these concentrations, whereas mercuric chloride would (White et al., 1973). However, there is the very important probability that cyanide would convert Cbl to the stable cyano form.

Artificial saliva was infused using a calibrated 4-channel peristaltic pump (Ismatec, Zurich) via the inlet port at the base of each vessel. The vinyl transmission tubing used for the input of saliva (Altec Ltd., Alton) was routinely replaced every 14 d.

Rumen contents used for inoculation were taken from rumen-fistulated sheep using a wide bore suction tube. The diets of the donors are described with the relevant experiment.

On the second day, the solid inoculum was replaced by a bag of the chosen ration. The procedure followed was that the vessel was disconnected from the drive mechanism, taken from the waterbath and the food container carefully removed; allowing time for fluid to drain back into the vessel. The bag containing the solid inoculum was taken out and placed inside a polythene bag (250 x 150 mm), washed with 40 ml artificial saliva, previously warmed to 39 °C, and the contents of the polythene bag mixed by gentle squeezing. The resultant "washings" were poured into a beaker and the washing repeated. The volume of combined washings could be measured and a sample taken if required; otherwise they were returned to the reaction vessel and the solid inoculum discarded. A new food bag was placed in the food container and the vessel closed. If required the vessel fluid was brought up to the overflow by injecting artificial saliva through the 3-way tap, "topping up", and the volume required was recorded. The flushing procedure was then performed as before. Vaseline (The Boots Co. Ltd., Nottingham) was occasionally used to improve the seal of the cover.

On subsequent days the food bag that had spent 2 days in the vessel was removed and a new bag of food introduceed. Identification was achieved by use of coloured plastic discs attached to the bags. Digested matter in the bags was analysed for apparent dry matter digestibility (ADMD), and latterly apparent organic matter digestibilities (AOMD) were also determined (p 97). Samples of effluent and those samples taken from within the

vessel, via the 3-way tap, were stored in capped, 18 ml polystyrene tubes (Gallenkamp and Co. Ltd., London). The volume of effluent was recorded daily, as was the input of artificial saliva. These values allowed dilution rates to be determined for each vessel.

To minimise contamination by Co (p 105), in Experiments 2-5, Rusitec apparatus was soaked in an alkaline laboratory detergent (Decon, 5 % v/v) and afterwards thoroughly rinsed in deionised, distilled water prior to assembly.

The concept of segregation in the rumen is well known and similarly the Rusitec can be defined in terms of compartments (Czerkawski, 1979). Compartment 1 is the free liquid in the vessel and is the easiest to define, since its volume and the concentration of substances can be measured directly; it is from this compartment that the effluent will be directly produced. Compartment 2 is the liquid associated with the solid digesta and the concentration of substances within can be determined by analysing the washings. However, the volume of this compartment is that removed by washing plus, that retained in the squeezed solid minus the volume of compartment 3. Compartment 3 is that liquid so closely associated with the solid matrix that it cannot be readily removed. Determination of this volume has been described (Czerkawski, 1979) and removal of this liquid, while possible by stringent washing of solid matter (Czerkawski, 1979), is difficult.

Cobalamin and total vitamin B12 analysis

Difficulties were anticipated in using established techniques for distinguishing between Cbl and analogues in analogue-rich samples from the Rusitec. Nevertheless, it was decided to begin with the techniques used by others for similar puposes.

Poteriochromonas malhamensis This assay, considered specific for Cbl (Ford, 1953), was tried initially. P.malhamensis Chen 933/1a was obtained from the Culture Centre for Algae and Protozoa, Cambridge. Considerable problems occurred with microbial contamination of the culture, but these were eventually overcome using stringent conditions of hygiene when sub-culturing. The linear standard curve (0-118 pmol Cbl/l) described by Ford could not be achieved, the best obtained was linearity over the range 0-59.0 pmol Cbl/l. Surprisingly, it was found that growth responses to samples from Rusitec increased substantially as the dilution factor increased and removal of the inhibitory effect of acetate was suspected. Acetic acid in excess of 17 mM has been found to inhibit growth (Ford, 1953) and this was far greater than the concentration produced by undiluted, Rusitec samples. Construction of standards with a series of acetic acid dilutions did not have any effect. Recoveries of added Cbl were extremely variable and always less than 100 %. These results may have been to due inhibition, by analogues, of the growth response to Cbl (Ford, 1958; 1959; Kamikubo and Hayashi, 1979), an effect which could be lessened by dilution (Ford, 1958). Since each dilution would require a

separate standard curve and a wide range of Cbl and analogues was expected, the technique was not persevered with.

Competitive protein binding radioassay Previous analyses for vitamin B12 at the Moredun Institute had been undertaken, on serum samples only, using the c.p.b. method of Lau et al. (1965); with the modifications that 0.5 N replaced 0.25 N hydrochloric acid and a 0.029 M sodium cyanide solution was incorporated. As other workers (Elliot et al., 1971; Rickard et al., 1975) had used similar methods for vitamin B12 analysis of rumen samples the c.p.b. method was evaluated for Rusitec samples.

The unknown, hydrochloric acid and sodium cyanide were mixed in equal volumes (1 ml) prior to heating (ca. 100 °C) for 30 m. The IF preparation was supplied by Amersham International plc, Amersham. A standard curve was constructed using Cbl in vitamin B12-free fluid. The results were as disappointing as those for the P.malhamensis assay. Poor recoveries of Cbl added to unknowns were obtained and once more vitamin B12 concentrations were apparently increased by dilution. Replacement of the 0.5 N by 0.03 N hydrochloric acid or an acetate buffer of pH 4.60 produced a higher pH for liberation, but no significant improvement in results.

Work by Kolhouse et al. (1978) suggested that the IF used was likely to be impure. An assessment of this IF preparation, by displacement of a known amount of labelled CN-Cbl with excessive amounts of dicyanide cobinamide,

TABLE 2.2 Solutions prepared for c.p.b. radioisotopic assay of cobalamin.

Extracting buffer

0.4 M sodium acetate titrated to pH 4.0 with 0.4 M hydrochloric acid. Sodium cyanide is added at 20 mg/l to the final solution.

Neutralising buffer

0.8 M glycine dissolved im 0.8 M sodium chloride and titrated to pH 10.0 with 0.8 N sodium hydroxide.

IF solution

Solution composed of 0.5 units/ml phosphate buffer, where the value of each unit was as supplied by Sigma Chemical Co. Ltd., Poole, Dorset. The phosphate buffer was 0.1 M potassium dihydrogen phosphate titrated to pH 7.50 with 0.1 M potassium hydroxide and containing 1 g bovine serum albumin /l of final buffer.

Albumin-coated charcoal

A 1 % (w/v) solution of bovine serum albumin in saline was added to a 5 % (w/v) aqueous solution of acid-washed, activated charcoal and stirred thoroughly for a minimum of 15 m prior to use.

N.B. Unless stated all chemicals were Analar grade supplied by BDH Chemicals Ltd., Poole, Dorset.

The phosphate buffer was found to have a maximum, working shelf life of 1 month.

suggested that cobalophilin constituted approximately 20 % of the binding capacity. With the use of the strong acid to ensure a low pH at liberation cobalophilin was probably the effective binder and even at the higher pH introduced for liberation its contribution could be substantial (p 70). Consequently, this assay would provide a measure of total vitamin B12, but because of the differences in binding affinities of different analogues to cobalophilin quantitative results could not be expected.

Competitive protein binding radioisotopic assay for cobalamin in rumen fluid

The rapidity of the c.p.b. radioassay compared to the microbial assay prompted an attempt to improve its specificity. This technique is essentially the method of Green (1980), in which binding occurs at pH 9.0 coupled with the use of a purified IF. The IF was a lyophilised preparation of porcine gastric mucosa (Sigma Chemicals Co., Poole) and was claimed to contain less than 5 % of the binder as cobalophilin. Hence binding of Cbl analogues should be minimal. Although a structural and possibly physiological difference in gastric juice IF to that in the mucosa has been suggested (Jacob et al., 1980).

One ml of Rusitec fluid was added to 2 ml of the extracting buffer (Table 2.2) in glass test tubes, mixed, the tubes loosely stoppered to prevent evaporation and placed in a boiling water bath (ca. 95 °C) for 30 m. An acetate buffer was used for liberation so that the same extract could be used in the L.leichmanii assay (p 91).

The Cbl concentration of an aqueous stock solution was determined spectrophotometrically at 351 and 550 nm (Merck Index, 1968) and a working solution, containing 13.8 nmol Cbl/l prepared in distilled, deionised water, was treated like the unknowns. All the tubes were allowed to cool prior to centrifugation at 50,000 g for 20 m at 4 °C to remove any precipitate and/or particulate matter. The supernatant (extract) was stored at -20 °C in capped, polystyrene containers prior to the binding stage of the assay.

All standards, unknowns and blanks were analysed in duplicate. Aliquots of "extract", upto 750 µl, and 0, 5, 10, 20, 40, 80, 120 and 160 µl "extracted" standard Cbl solution were dispensed into 3.5 ml polystyrene assay tubes (Sarstedt U.K. Ltd., Leicester) and the volume adjusted to 750 µl with the extracting buffer, which had been diluted 2:1 with water. Completion of the assay in small volume polystyrene tube allowed for ease of operation in centrifugation. The quantities of standard were 0, 23.1, 46.1, 92.3, 125, 369, 554 and 738 fmol (1 pmol = 1000 fmol). These corresponded to 0-1000 pg Cbl/tube. "Blanks" were prepared with a Cbl solution in the diluted extracting buffer in excess of 7.38 µmol Cbl/l, i.e. sufficient to saturate the specific binding capacity of the assay system. These blanks allow correction for natural background radiation and nonspecific binding of Cbl. The values for these blanks were of the same order, i.e. 9 c.p.s., as those for blanks composed by substituting phosphate buffer alone for the IF in phosphate buffer solution.

0.5 ml aliquots of a 57Co-CN-Cbl (Amersham International plc, Amersham) working solution, in saline, of 66.4 pmol Cbl/l were dispensed to each tube, together with 0.5 ml neutralising buffer and 0.25 ml IF solution (Table 2.2). Stored at -20 °C, in aliquots of 10 units in the phosphate buffer (Table 2.2), there was no reduction in the binding capacity of the IF for periods up to 9 months. The binding capacity of IF was determined by serial dilutions of IF added to the zero standard and that concentration binding 40-60 % of the 33.2 fmol 57Co-CN-Cbl added was used in the assay. All the tubes were then agitated thoroughly using a vortex mixer and allowed to stand at room temperature for 1 h.

One ml of albumin-coated charcoal (Table 2.2) was added to each tube and after 10 m they were centrifuged at 3,800 g for 20 m at 4 °C. The supernatant was decanted into counting vials and counted in a Nuclear Enterprises 8311 gamma counter with a thallium-activated, sodium iodide crystal. In addition to these samples, 0.4 ml of the 57Co-CN-Cbl working solution was pipetted into counting vials to ascertain the total radioactivity used in the assay. The difference in volume between these "totals" and the decanted supernatant did not influence the counting.

The proportion (y) of added radioactivity in the supernatant was determined for each standard after correction for blanks and the relationship to the amount of Cbl standard derived by least squares linear regression computation (Green, 1980). Cbl concentrations for the unknowns were then determined from this equation. Drift

within the assay was not noticeable in repeated standards or unknowns.

Rusitec "liquid" samples taken for analysis will contain bound, and possibly free, vitamin B12 in the liquid phase. They will also contain food particles, viable microbes and dead microbes, intact and lysed. Microbes will adsorb, then absorb the vitamin. Adsorption of Cbl by E.coli has been found to be rapid, saturable, independent of temperature over the range 15-35 °C and maximal about pH 6.0 (DiGirolamo and Bradbeer, 1971) and it can occur onto heat-killed bacteria (Giannella et al., 1969). Absorption by the same organism has been shown to be slower (30-60 m), temperature dependent and susceptible to inhibitors of energy metabolism, e.g. 10 mM potassium cyanide, (DiGirolamo and Bradbeer, 1971). 57Co-CN-Cbl was shown to be adsorbed by microbes and/or food particles (10-60 % dose added) when Rusitec fluid was incubated at 20 °C or 37 °C, but there was no difference in incubating for 5 or 60 m. This labelled Cbl was completely released when subjected to the liberation procedure with the extracting buffer. Such a phenomenon suggests that adsorption, but not absorption, was occurring and that the obligate, rumen anaerobes may be inhibited by the aerobic conditions of this incubation. Coated charcoal was found to absorb all the labelled CN-Cbl from the treated sample, after it had undergone liberation with the extracting buffer. It was assumed that adsorbed, but not absorbed, vitamin B12 was made available for subsequent analysis; in addition to the bound and free non-microbial vitamin B12.

Assay validation The problems of vitamin B12 analysis discussed previously demanded that an evaluation of the assay be undertaken.

The pH of the assay tube contents after addition of 57Co-CN-Cbl, neutralising buffer and the IF solution was 9.20-9.60; this is within the optimum range for binding. Specificity of the binder was checked by addition of 185 fmol dicyanide cobinamide to dilutions of an unknown, such an addition ensured proportions of analogue in total vitamin B12 far greater than those encountered in normal rumen samples. These additions increased the values of the unknown, and the mean increase was 9.59 %; however, this effect could be explained by the 5 % cobalophilin contamination of the binder. If the affinity of an analogue for cobalophilin is greater than that of Cbl, then when sufficient analogue is present to saturate this binder the c.p.s. will decrease by no more than 5 % in any sample, if nonspecific binders are present as 5 % or less of the total binder. The effect of this will be proportionally greater as the higher end of the standard range is reached. However, cobalophilin has been shown to bind cobinamide with an affinity equal to that for Cbl at pH 8.0 (Allen et al., 1978b), suggesting that any errors produced by the presence of analogues would be less than the theoretical maximum, as binding in this assay occurs at pH 9.20-9.60. Similarly, additions of dicyanide cobamide to a number of standards did not substantially affect the results.

Interference by factors other than vitamin B12 was checked by preparing vitamin B12-free Rusitec fluid. Use of the

extracting buffer liberated the vitamin which then remained unbound and was removed by uncoated charcoal. Increments of this fluid were incorporated into the standards, but found to have an insignificant effect. Vitamin B12-free fluid was not therefore added routinely to standards, although it was required in the microbial assay (p 91).

Inter-assay variation was ascertained by repeatedly analysing a pooled sample as a quality control. This required a 6-fold dilution to produce a value in the central region of the standard range. All unknowns were then corrected for day to day variation using the ratio $\bar{Q.C.} : Q.C.$; where,

$\bar{Q.C.}$ = mean value of the known quality controls.

Q.C. = quality control on day x.

The size of the correction factor diminished as experience of the technique grew. For the few days when the quality control was omitted a value was derived from the equation

$$\frac{Q.C}{\bar{Q.C.}} = \frac{b\ pool}{b}$$

where, b pool=regression coefficient for standard curve derived from pooled, standard values

b=regression coefficient for standard curve on day x.

Although retrospective correction was necessary for all samples the technique still allowed for the effect of treatments and the behaviour of vessels to be monitored. Intra-assay variation was slight, with the coefficient of variation, for unknowns analysed at a single dilution,

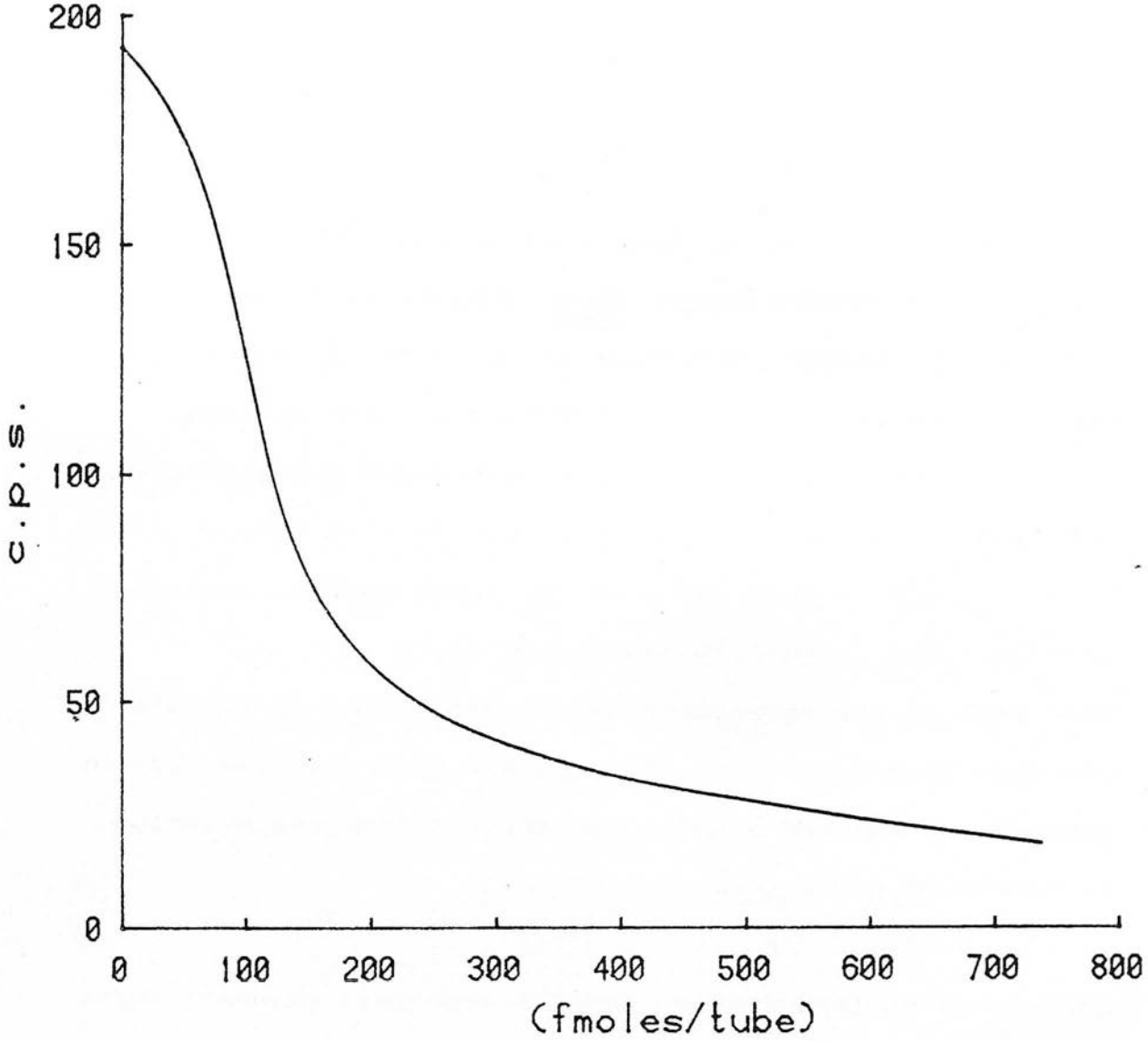


FIG. 2.2 Typical standard curve for the cobalamin radioassay, before transformation.

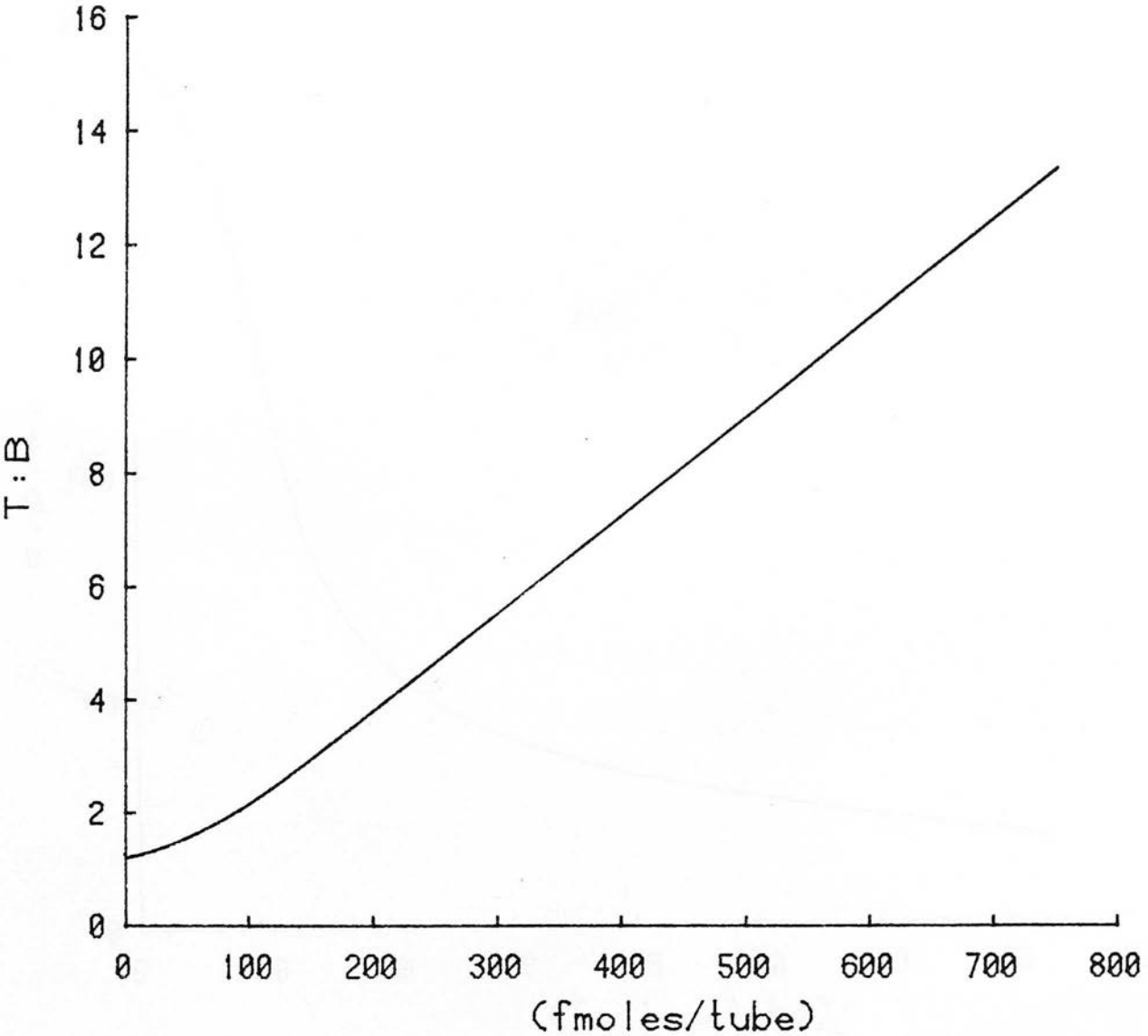


FIG. 2.3 Typical standard curve for the cobalamin radioassay, after transformation.

being less than 3 %. Recoveries of CN-Cbl additions to Rusitec fluid, prior to liberation, were 95 and 97 %. Recoveries of CN-Cbl added to unknowns, after liberation, covered the range 82-140 %; with a mean recovery of 106 % (n=19). Serial dilutions of unknowns were made and covered the range 20-648 fmol Cbl. The coefficients of variation of these series, after correction for dilution, ranged from 3.6-16 %, mean 7.5 %. Thus there was no evidence of the anomalous dilution effects of the previous methods. Most of the unknowns required dilution as the Cbl concentrations were in excess of the highest standard. Sensitivity of the assay was estimated by use of a radioimmunoassay computer program (M.R.C., Edinburgh). This used a logit-log transformation of the standards and computed a minimum detectable value using the variance of the regression. The mean value for 6 standards curves, thus treated, was 50 fmol. Results for the validation of this assay were comparable to those of Green (1980).

The standard range was 0-738 fmol Cbl/tube. A typical standard curve before and after transformation is shown in Figs. 2.2 and 2.3. While correlation coefficients for the regression line of the standards were extremely good (r normally > 0.99), examination of the uppermost standards (Fig. 2.2) indicated that the upper limit be lowered to 500 fmol Cbl/tube. In addition, the deviation from linearity that occurred in the range 0-100 fmol Cbl/tube (Fig. 2.3) suggested that the lowest unknown should contain 100 fmol Cbl/tube. This is in accordance with previous work with IF in showing that it does not obey proportional dilution principles (p 72).

Lactobacillus leichmannii assay for total vitamin B12

The L.leichmannii microbial assay was chosen for the determination of total vitamin B12 (Cbl plus analogues) because,

a) it is responsive to the predominant analogues separated from rumen contents (Gawthorne, 1970a) (Table 1.6),

b) it requires an incubation period of less than 24h,

c) the required assay media is available commercially,

d) it has been used previously for rumen fluid analysis (Dawbarn et al., 1952; Smith and Marston, 1970a), although values for rumen contents are greater when either the E.coli plate or tube assays are used.

L.leichmannii (ATCC 7830), supplied by the Torry Research Station, Aberdeen, was maintained by daily transfers of 100 µl sub-cultures to 10 ml sterile micro inoculum broth (Difco Laboratories, Surrey) incubated at 37 °C in darkness.

The assay technique was a modification of that recommended by Difco Laboratories. Vitamin B12 was extracted as described for the c.p.b. radioisotopic assay (p 84). Vitamin B12-free assay medium (Difco Laboratories, Surrey) was prepared by dissolution of the dehydrated material in distilled, deionised water, this water being used throughout the project unless otherwise stated, and dispensed in 5 ml aliquots into McCartney bottles. All standards and unknowns were prepared in duplicate. The stock solution of Cbl (p 85) was used to prepare an aqueous working solution of 73.8 pmol Cbl/l (100 ng Cbl/l). Aliquots of 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 4.5 ml were

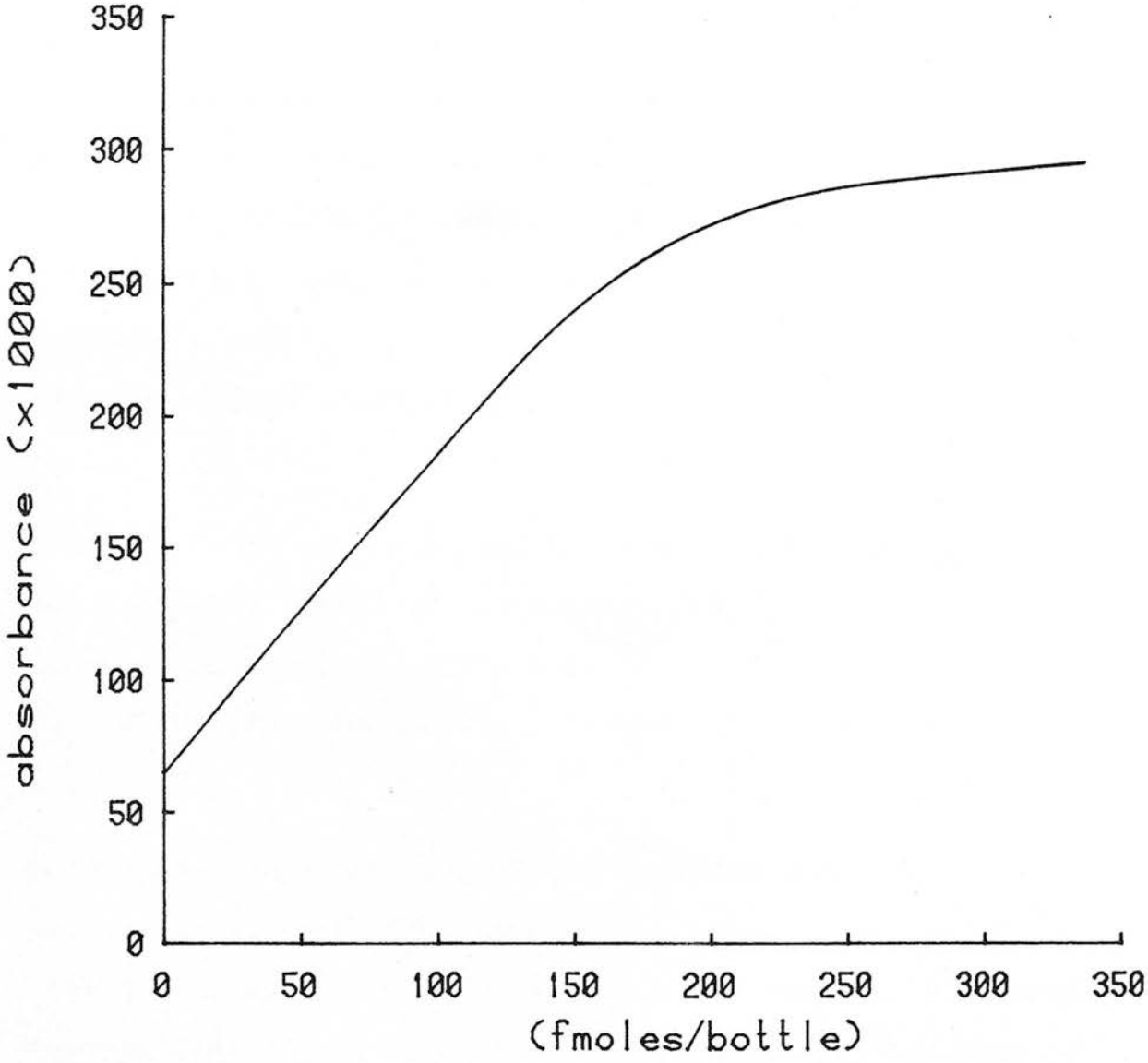


FIG. 2.4 Growth response curve for the Lactobacillus leichmannii microbial assay.

used to produce standards of 0, 36.9, 73.8, 111, 148, 222, 295 and 332 fmol Cbl/bottle. Vitamin B12-free Rusitec fluid was added to the standards in volumes that would be required of the unknowns, because inclusion of it had been found to increase the growth of the assay bacteria. Appropriate volumes of the unknown extracts were then added to 5 ml assay media in McCartney bottles and the volumes made upto 10 ml with water. After securely capping, the bottles were sterilised by autoclaving at 121 °C (6.90×10^4 Pa) for 2 m.

The inoculation was prepared from a culture grown for 24 h in the micro inoculum broth. This culture was washed 4 times in 10 ml sterile assay media and 200 µl of this washed suspension diluted in 20 ml sterile assay media; of this 50 µl was used as the inoculum for each bottle. Care was taken at all times to maintain sterile conditions, e.g. flaming. All the bottles were then incubated at 37 °C in darkness for 16-24 h. Growth responses were measured as the turbidity of the culture at 550 nm.

The zero standard showed slight growth, assumed to be due to factors other than vitamin B12 (p 57), and was used as the blank. A response curve for the standards is shown in Fig. 2.4. The linear section was utilised for determining vitamin B12 concentrations, a straight line being fitted by the least squares method of regression. A pooled sample was analysed throughout to measure inter-assay variation. This was large and quality control was achieved in the manner described for the c.p.b. assay using the mean value of this unknown. While unknowns are expressed in terms of

TABLE 2.3 Operatng conditions for VFA analysis.

Parameter	Method 1	Method 2
Col. temp. (°C)	130	135
Inj. temp. (°C)	170	170
Det. temp. (°C)	175	175
Nitrogen flow rate (ml/m)	40	45
Sample volume (µl)	0.3	0.6

molar levels of analogues this is not strictly accurate since pure solutions of Cbl were used for the standards. Consequently, more detailed validation of the assay was not undertaken.

Volatile fatty acid analysis

Samples for VFA analysis were centrifuged at 2,000 g for 15 m to remove particulate matter, diluted 4:1 with 1.5 M metaphosphoric acid and mixed thoroughly. They were then left to stand for a minimum of 30 m at 4 °C, after which they were centrifuged at 2,000 g for 15 m and the supernatant retained. Unknowns, standards and water blanks were treated in a similar manner and stored in glass vessels at -20 °C. All glassware used in this project was washed with an aqueous EDTA solution (0.5 % w/v). VFA analysis of rumen and Rusitec fluid was performed by gas-liquid chromatography on a G.C.D chromatograph (Pye Unicam Ltd., Cambridge).

Method 1 Initially the separations were made with 10 % SP-1200/1 % H PO on 80/100 chromosorb W AW packing (Supelco Inc., U.S.A.) in a 6 ft x 2 mm i.d. glass column. VFA upto C5 eluted within 5 m with good separation, when the conditions detailed in Table 2.3 were adhered to. The technique is detailed in Technical Bulletin 749A (Supelco Inc., U.S.A., 1975) and injections were with a 1 µl syringe (S.G.E. Pty. Ltd., Australia).

Determination of VFA in the unknown was by comparison of peak height measurements with an external standard in water composed of acetic, propionic, i-butyric, n-butyric,

i-valeric and n-valeric acids at concentrations of 52.4, 13.4, 5.39, 10.9, 4.22 and 4.60 mM respectively (Supelco Inc., U.S.A.). The 1.5 M metaphosphoric acid which was used for deproteinisation and as a "tail reducer" (Ottenstein and Bartley, 1971b) was replaced by 1 M orthophosphoric, after Experiment 1, to attain more reproducible acetate peaks (J. Czerkawski, pers. commun. It also had the advantage of being an easier compound to handle. However, the reproducibility for the acetate and propionate peaks was still unsatisfactory. Overloading of the column was considered as a possible cause (Ottenstein and Bartley, 1971a), but reducing the sample size did not improve the results. The speed and precision of injection was improved by incorporating a manual injection unit (Clandon Scientific, Aldershot), but the problem persisted.

Rapid elution had enabled a large number of unknowns to be analysed in a day. Consequently, there was the possiblity of an accumulation of carbonaceous non-volatiles at the inlet of the columns; deposits which adsorbed VFA and caused tailing (Ottenstein and Bartley, 1971b). A replaceable glass inlet can be used to trap these substances; but no supplier of these, for the column used, could be found. Instead the top few centimetres of the column, as well as the phosphoric acid-treated glass wool plug (Supelco Inc., U.S.A) were replaced daily and the column "conditioned" overnight at 190 °C to remove volatile compounds. This technique was used to analyse samples upto the completion of Experiment 2.

TABLE 2.4 Validation of VFA analysis using "10 % FFAP on 100/120 Diatomite CQ" column.

	VFA						
	Acetic	Propionic	i-butyric	n-butyric	i-valeric	n-valeric	
Intra-assay c.v.(%)							
Standards	5.23	4.60	4.42	4.48	4.57	5.93	n=22
Pooled sample	4.79	4.11	-	4.63	4.31	4.87	n=25
Inter-assay c.v.(%)							
Pooled sample	9.33	10.2	-	14.0	17.4	24.9	n=16
Recovery of added VFA(%)	101	100	98.2	110	110	149	n=2
c.v. (%) of corrected values for 8-fold dilution series of unknown	5.59	5.08	-	5.08	10.9	36.0	n=4

Method 2 For experiments 3-5, 10 % FFAP on 100/120 Diatomite CQ packing in a 1.5 m x 4 mm i.d. glass column (Pye Unicam Ltd., Cambridge) was used. VFA upto C5 were eluted within 25 m under the conditions given in Table 2.3. Reproducibility was better with this column and the top of the column had only to be replaced when the performance was seen to deteriorate (ca. every 300 assays) and accumulation of carbonaceous volatiles was not so influential. The same standards as were employed for Method 1 were used with this column.

For Experiment 5, determination of concentrations was calculated automatically using a reporting integrator (Hewlett Packard, U.S.A.). Estimation was by area for individual acids, compared to the external standard.

Mean percentage differences for three unknowns calculated by integration compared to values determined by peak height were -4.29, -2.02, +2.8, -1.51, -5.72 and +7.50 % for acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric acids respectively. Validation of the assay is detailed in Table 2.4. The atypical behavior of n-valeric acid for the recovery, dilution and inter-assay variation was considered to be due to the high elution time of n-valeric acid and the subsequent poor peak shape.

Trace element analysis

Cobalt Co analyses of plants and soils were kindly undertaken in the Trace Element department of the East of Scotland College of Agriculture. The method they used will be briefly described.

Soil samples were dried at 100 °C and ground before use. Extractable soil Co was measured using 0.5 N acetic acid as extractant and complexing with 2-nitroso-1-naphthol in the presence of sodium citrate and hydrogen peroxide, prior to measurment by flameless atomic absorption specrophotometry at 240.7 nm. Total soil Co required digestion of the soil in aqua regia and dissolving in 6 N hydrochloric acid. No concentration stage was required for measurement by flame atomic absorption at 240.7 nm.

Initial destruction of organic matter in plant samples was achieved by 2 dry ashings at 450 °C, the first followed by wet ashing in 16 N nitric acid. The digest was then dissolved in 0.5 N acetic acid, and Co complexed with 2-nitroso-1-naphthol using the procedure for extractable soil Co.

Copper, iron and zinc Analyses for these elements were undertaken on Rusitec effluent samples from Experiment 4, to measure the release of these elements from soils, and on the low Co hay(2) used as the substrate. Glassware used in iron and zinc determinations had a second wash in 5 % nitric acid. Digestion of 5 ml effluent was accomplished using 5 ml perchloric/citric acid mix (1:4 v/v) on heating blocks described by Thompson and Blanchflower (1971). The dry digest was then made up to 5 ml with 5 % hydrochloric acid and assayed for each element on an I.L. 151 atomic absorption spectrophotometer against commercially prepared standards (B.D.H., Poole) diluted with 5 % hydrochloric acid and using water as a blank. The wavelengths for copper, zinc and iron determinations were 324.7, 213.7 and 248.3 nm respectively.

Digestibility and pH measurements

Digested rations from Rusitec were dried to constant weight at 100 °C for 48 h and the value for the DM fed used in determining the (ADMD).

$$\text{ADMD} = \frac{\text{DM fed} - \text{undigested DM}}{\text{DM fed}}$$

The apparent organic matter digestibility (AODM) was calculated in a similar manner to ADMD. Ashing of the digested ration in a muffle furnace at 500 °C for 20 h determined the undigested OM and the value for OM in the original ration was used in the equation.

pH values for Rusitec liquor were determined, using a calibrated meter (Corning Scientific Instruments, U.S.A.), within 45 m of sampling.

TABLE 3.2 Experiment 1. Experimental procedure for Rusitec vessels 1-4 in terms of dry matter input (g/d) of low cobalt hay(1) and supplementation with cobalt (nmol/d).

	Vessels 1, 3 and 4			Vessel 2	
	DM (g/d)	Co supplement		DM (g/d)	Co supplement
Period		Vessel			
1	7		-	7	-
2	7	1 3 4	14.3 28.5 57.1	7	28.5
3	10.5		14.3 28.5 57.1	7	57.1

CHAPTER 3

EXPERIMENT 1. THE SUITABILITY OF RUSITEC FOR STUDIES OF VITAMIN B12 SYNTHESIS

Introduction

Previous *in vitro* studies of rumen vitamin B12 synthesis have been short-term (Gawthorne, 1970b) and some have used single species cultures (Dryden *et al.*, 1962; National Institute for Research in Dairying, 1964). Although continuous cultures would be expected to be closer simulations of the rumen environment, the suitability of Rusitec for this purpose required investigation. The first experiment examined inter-day and inter-vessel variation using unsupplemented and Co supplemented hay. Dose: response curves for vitamin B12 synthesis were sought and the influence of DM input was also investigated.

Experimental procedure

To implement these studies a mixed grass hay(1) (Table 3.1) of low Co content (543 nmol Co/kg DM= 0.028 mg/kg DM) was obtained from East Nisbet farm, Crailing, nr. Jedburgh. Inoculum for Rusitec was taken from the rumen contents of a freshly slaughtered Blackface sheep that had been given Ruminant A, a complete, processed diet (Wainman *et al.*, 1970). The treatments employed in this experiment are clarified in Table 3.2. In period 1 the low Co hay(1) was "fed" for 54 d at 7 g DM/d to establish resting (unsupplemented) values. After 11 d a leak developed in 1 of the 4 vessels (vessel 2). It was repaired and

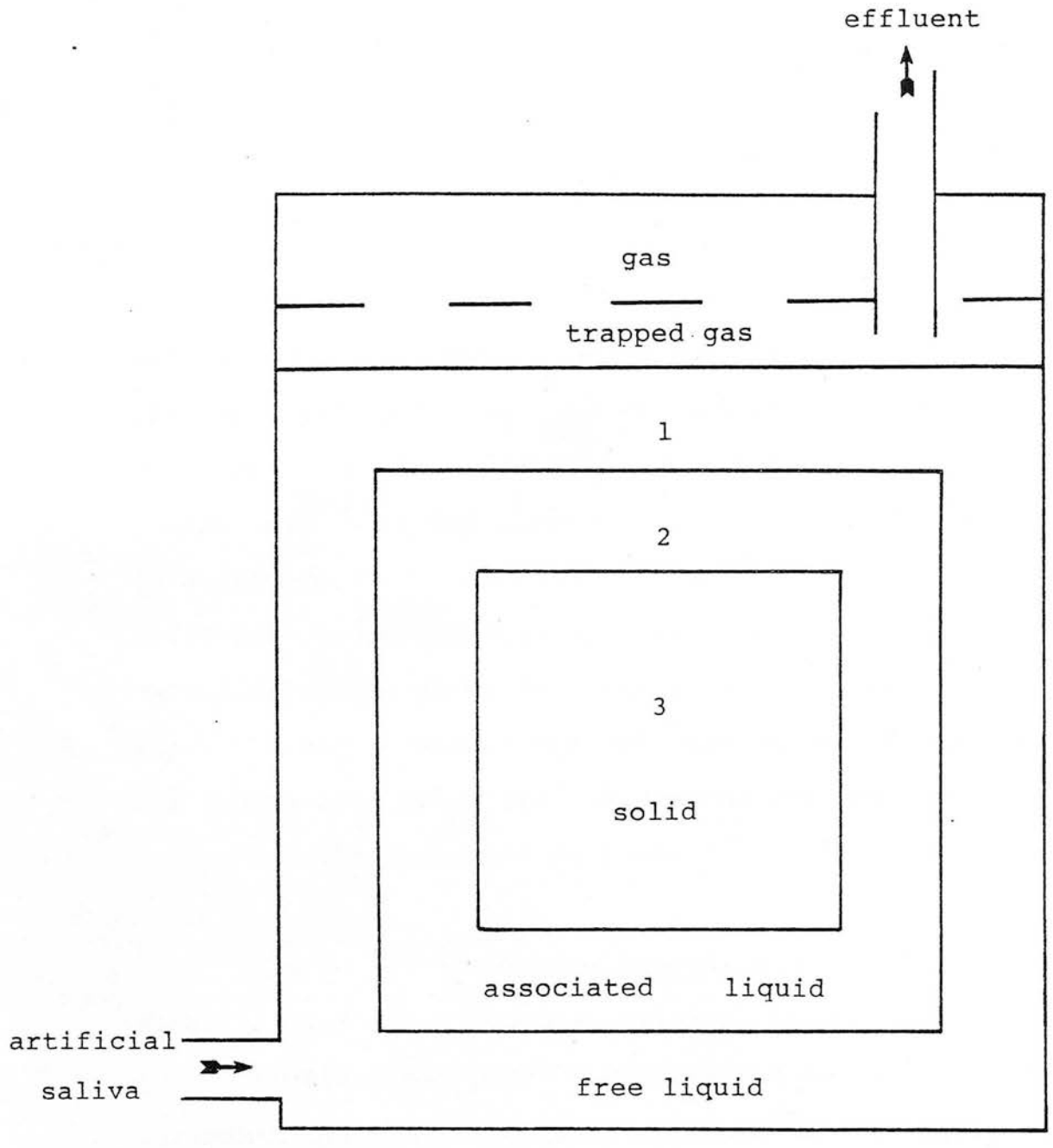


FIG. 3.1 Schematic representation of the three compartment model in Rusitec.
(Compartments: 1= free liquid,
2= liquid associated with solid matter,
3= liquid very closely associated with solid matter and which cannot be readily washed out)
(Czerkawski and Breckenridge, 1979b)

reinoculated using rumen contents obtained via a fistula from another Blackface sheep given the Ruminant A diet, and then treated like the other vessels during this period.

In period 2 (d 55-76), Co was infused via the artificial saliva (Table 2.1), using dilutions of a standard Co nitrate solution (B.D.H., Poole) to provide 14.3, 28.5, 28.5 and 57.1 nmol Co/d, into vessels 1, 2, 3, and 4 respectively (equivalent to 0.12, 0.24, 0.24 and 0.48 mg Co/kg hay DM); this was to establish the dose: response curve for vessels 1, 3 and 4. Vessel 2 had developed a different fermentation compared to the other vessels and from d 55 it was given the same Co supplement as vessel 3 to determine if vitamin B12 production was different.

In period 3 (d 77-99), the hay input was increased to 10.5 g DM/d for vessels 1, 3 and 4, with no increase in Co supplementation. The hay input in vessel 2 was maintained at 7 g DM/d and the Co supplement increased to 57.1 nmol/d to obtain a dose: response curve for this atypical vessel.

During this experiment samples of vessel fluid from compartment 1 (Fig. 3.1) were taken via the 3-way tap (Fig. 2.1), immediately prior to feeding. Analyses of these samples for Cbl and VFA were undertaken and output values for the vessel were derived from the product of analyte concentration and the daily volume of effluent. The term "output" is used throughout for the productivity of the system as determined by sampling from within the vessel, prior to feeding. This procedure for measuring the productivity of the system was adopted throughout

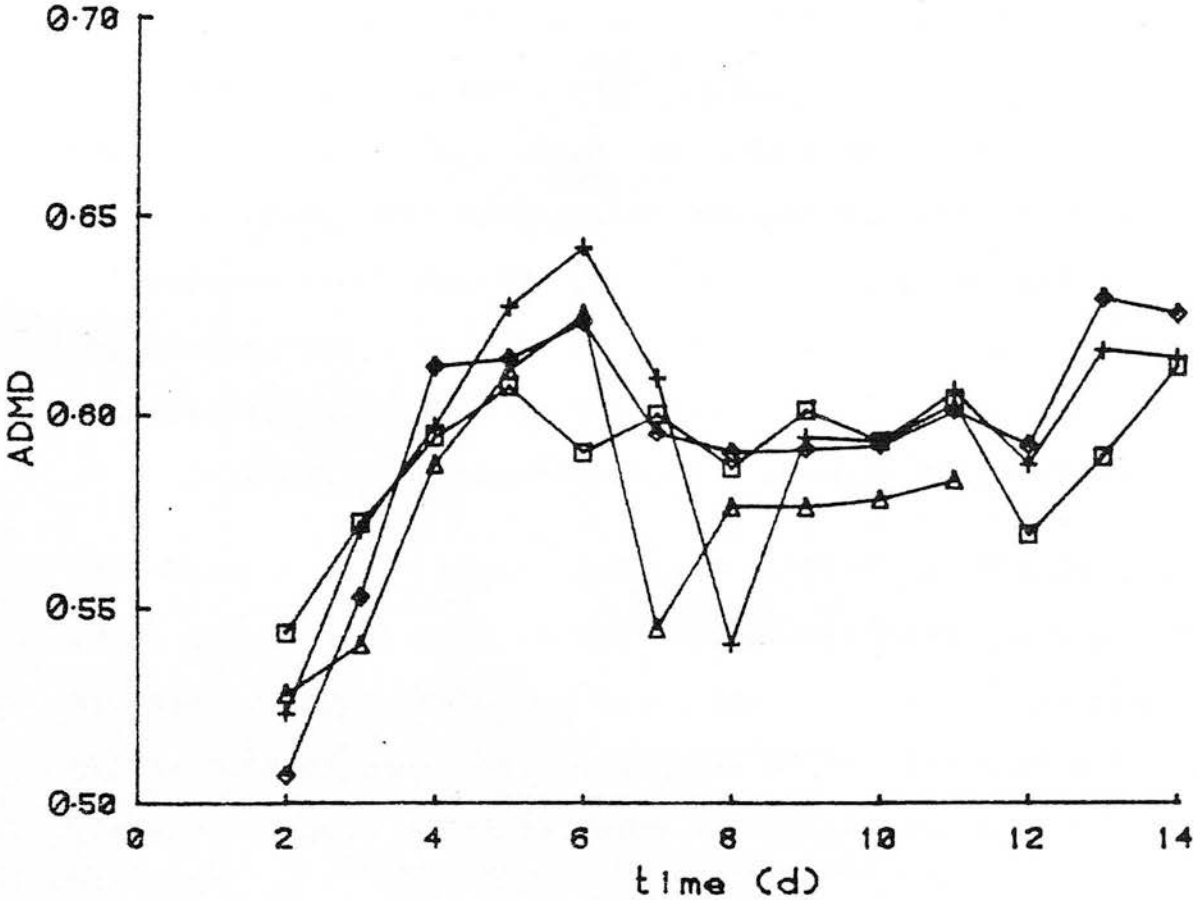


Fig. 3.2 Experiment 1. Daily variation of ADMD for low cobalt hay(1) given to Rusitec vessels 1-4 at 7 g DM/d.
(vessels: 1= +, 2= △, 3= □, 4= ◇)

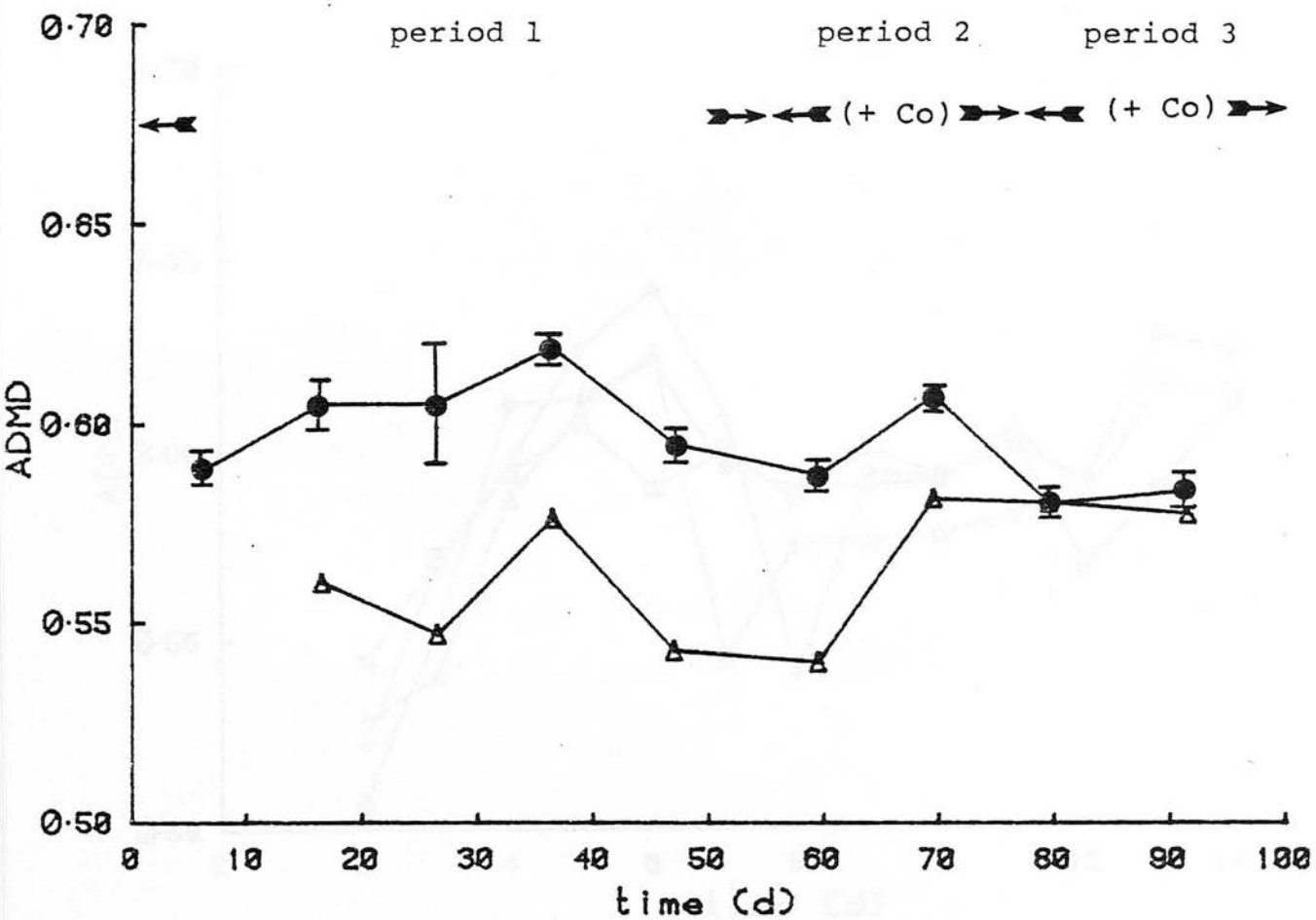


FIG. 3.3 Experiment 1. ADMD values (mean ± s.d.) for vessels 1, 3 and 4 in 4-11 d sub-periods (●), compared with those for vessel 2 (△), when all vessels were given the low cobalt hay(1) with or without cobalt (see Table 3.2 for details).

TABLE 3.3 Experiment 1. Molar proportions of total VFA (%) and acetate: propionate ratios for the fermentation in vessel 2 compared to those in vessels 1, 3 and 4 (mean ± s.d.). Values for each vessel derived from sampling on d 48 and d 50.

	Vessel	
	1,3 and 4	2
Acetic acid	67.5 ± 0.200	52.2
Propionic acid	17.5 ± 0.529	25.0
Total butyric acid	9.57 ± 0.361	10.5
Total valeric acid	5.52 ± 0.160	12.4
Acetate: propionate	3.86 ± 0.117	2.09

TABLE 3.4 Experiment 1. Effect of increasing the input of hay on mean cobalamin outputs expressed in three forms, when supplemented with cobalt at different levels in vessels 1, 3 and 4.

	Nominal cobalt supplement (nmol/d)			
	0 ± s.d.	14.3	28.5	57.1
Vessel	1, 3 and 4	1	2	3
Hay input (g DM/d)				
a) pmol Cbl/d				
7	1510 ± 99.5	1670	1780	2510
10.5	-	1200	1260	1760
b) pmol Cbl/d/g DM				
7	215 ± 14.2	238	254	359
10.5	-	171	180	245
c) pmol Cbl/d/g DM digested				
7	357 ± 26.4	387	407	579
10.5	-	191	199	275

Experiments 1 and 2. In Experiments 3-5, the amount of analyte in the effluent was used to derive "production" values (Ch. 8, p 208). A further check on the stability of the fermentation was maintained by determination, on alternate days, of the ADMD of the feed. The dilution rate varied from 0.90-0.75 /d during the experiment, due to an inefficient pump, but these rates were within the limits set by Czerkawski and Breckenridge (1977).

Results

Resting values with an unsupplemented hay diet (period 1)

After inoculation, ADMD increased steadily in all vessels for 6 d (Fig. 3.2). While variation in ADMD did subsequently occur, the changes in the 4 vessels were usually synchronised and the c.v. for ADMD on any single day was never greater than 5 %. A low ADMD became noticeable after reinoculating vessel 2 and was evident for most of the experiment (Fig. 3.3).

The VFA molar proportions for vessels 1, 3 and 4 (Table 3.3) were typical of those produced by a roughage diet (McDonald *et al.*, 1977); whereas in vessel 2, after reinoculation, there was far less acetate and more propionate (Table 3.3). Therefore, vessel 2 will be considered separately.

The Cbl output (mean ± s.d., n=3) from the low Co hay(1) in vessels 1, 3 and 4 was 1,510 ± 99.5 pmol/d (Table 3.4), with a conversion efficiency of Co into Cbl of 39.6 ± 2.65 % (mean ± s.d., n=3); when,

$$\text{conversion efficiency} = \frac{\text{Co in Cbl}}{\text{Co in hay}} \times 100\ \%$$

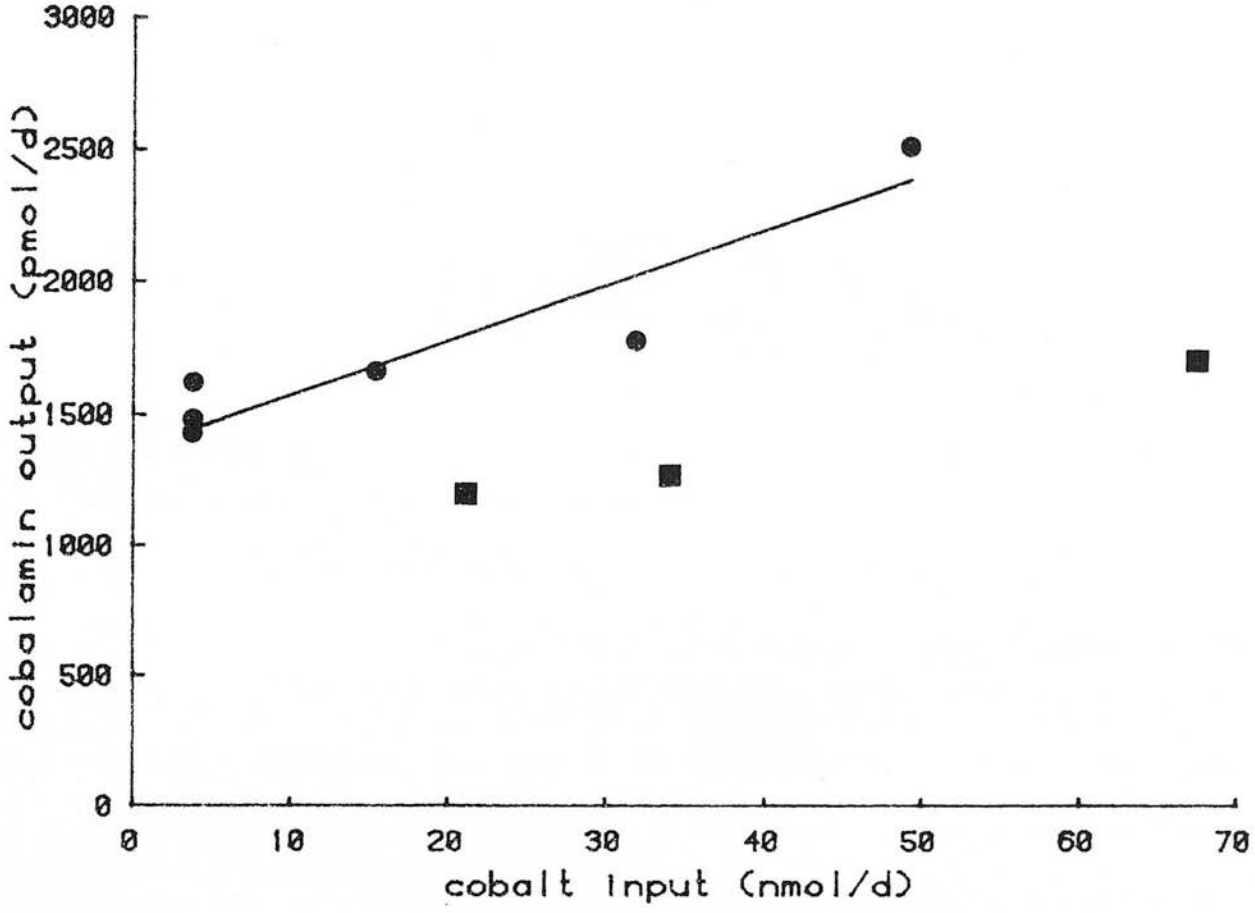


FIG. 3.4 Experiment 1. Cobalamin output from vessels given low cobalt hay(1) at 7 g DM/d (●) and 10.5 g DM/d (■), when unsupplemented and supplemented with cobalt.

TABLE 3.5 Experiment 1. Mean VFA synthesis in vessels 1, 3 and 4 when low cobalt hay(1), given at 7 g DM/d, was unsupplemented (period 1, d 48 and d 50), supplemented with cobalt (period 2, d 73 and d 75) and when the hay input was increased to 10.5 g DM/d and the same cobalt supplements retained (period 3, d 97 and d 99). Values from the two sampling days in each period were meaned and these values from the three vessels used to derive the means, with s.d. in parentheses.

VFA molar proportions (%)

	Acetic	Propionic	Total butyric	Total valeric
Period 1	67.5 (0.200)	17.5 (0.529)	9.57 (0.361)	5.52 (0.160)
Period 2	66.7 (0.666)	19.5 (0.643)	8.51 (0.577)	5.13 (0.0819)
Period 3	65.2 (0.265)	19.0 (0.0577)	10.6 (0.0577)	5.16 (0.366)

	Acetate: propionate
Period 1	3.86 (0.117)
Period 2	3.44 (0.122)
Period 3	3.44 (0.0289)

VFA output (mmol/d)

	Total	Acetic	Propionic	Total butyric	Total valeric
Period 1	60.6 (2.87)	40.9 (1.80)	10.6 (0.400)	5.80 (0.461)	3.35 (0.129)
Period 2	60.3 (0.819)	40.2 (0.794)	11.7 (0.513)	5.13 (0.316)	3.09 (0.0153)
Period 3	100 (0.577)	65.4 (0.346)	19.1 (0.153)	10.7 (0.000)	5.17 (0.369)

TABLE 3.6 Experiment 1. VFA outputs (mmol/d/g DM digested) for vessels 1, 3 and 4, given low cobalt hay(1) and supplemented with cobalt at two DM inputs. (Periods: 1= 7 g DM/d, 2= 7 g DM/d + cobalt supplement, 3= 10.5 g DM/d + cobalt supplement)

		Vessel		
	Period	1	3	4
Total VFA	1	14.5	14.8	13.7
	2	14.2	13.8	13.7
	3	15.9	15.9	16.1
Acetic acid	1	9.81	9.95	9.28
	2	9.49	9.27	9.06
	3	10.3	10.4	10.5
Propionic acid	1	2.61	2.49	2.43
	2	2.86	2.66	2.60
	3	3.04	3.03	3.03
Total butyric acid	1	1.37	1.47	1.28
	2	1.16	1.13	1.27
	3	1.70	1.69	1.72
Total valeric acid	1	0.782	0.822	0.778
	2	0.719	0.703	0.714
	3	0.886	0.806	0.777

N.B. It should be noted that the values for the mmol VFA/d/g DM digested were greater than the theoretical maximum derivable from hexose; all the values appear to have been overestimated by a similar margin and the comparisons made within the experiment should remain valid. For the remaining experiments the values for mmol VFA/d/g DM digested were acceptable.

Variation in Cbl output was large; the c.v. between vessels, for the 2 d upon which analyses were undertaken (d 48 and d 50), being 13.3 and 23.4 % compared to 0.220 and 1.63 % respectively, for ADMD.

Effects of cobalt supplementation upon fermentation (period 2)

Cobalamin synthesis Vessels 1, 3 and 4 will again be considered separately. Supplementation with Co increased Cbl output in all 3 vessels (Fig. 3.4, Table 3.4). Outputs of Cbl expressed in pmol Cbl/d (y) were proportional to Co input in nmol Co/d (x), as shown in Fig. 3.4, and this relationship was described by the regression equation:

$$y=19.5x + 1400 \qquad r=0.931 \qquad d.f.=4$$

The slope of x in this equation indicated that the overall trend was for 1.95 % of the added Co to be incorporated into Cbl.

VFA synthesis Supplementation of the low Co hay(1) with Co was accompanied by a significant ($p<0.05$) increase in propionic acid proportions plus a decrease ($p<0.05$) in the total valeric acid proportions and the acetate: propionate ratio (Table 3.5), as judged by the paired t-test, when results for Co supplementation were pooled and compared with the unsupplemented values. Treated similarly, VFA outputs were significantly increased for propionic acid, in both absolute ($p<0.01$) (Table 3.5) and acid /g DM digested ($p<0.05$) (Table 3.6) terms, and significantly decreased ($p<0.05$) for total valeric acid /g DM digested (Table 3.6).

TABLE 3.7 Experiment 1. Mean ADMD for vessels 1-4.
(Periods: 1= unsupplemented hay, 7 g DM/d,
2= cobalt supplement + hay, 7 g DM/d,
3= cobalt supplement + hay; 10.5 g DM/d
in vessels 1,3 and 4, 7 g DM/d in vessel 2)

	Vessel				
	1	2	3	4	Mean (1, 3 and 4) ± s.d.
Period					
1	60.7	55.8	60.0	60.5	60.4 ± 0.361
2	59.5	55.4	59.6	59.7	59.6 ± 0.100
3	58.4	58.2	58.5	58.5	58.5 ± 0.0577

Effects of an increased hay input upon fermentation during cobalt supplementation (period 3)

Cobalamin synthesis The increase in hay input from 7 to 10.5 g DM/d significantly reduced Cbl output at all levels of Co supplementation ($p<0.05$), as shown in Table 3.4. Consequently, the efficiency of incorporation of Co into Cbl was less at this higher DM input.

VFA synthesis and ADMD Comparison of periods 2 and 3, by paired t-test, showed that VFA molar proportions were only significantly ($p<0.05$) altered for total butyric acid, which increased (Table 3.5). However, significant increases occurred in all the VFA outputs when the hay input was increased (Table 3.5); the levels of significance being: $p<0.001$ for total VFA and acetic acid, $p<0.01$ for propionic and total butyric acids and $p<0.05$ for total valeric acid. ADMD underwent a small but significant ($p<0.01$) reduction from 59.6 ± 0.100 to 58.5 ± 0.0577 with the increased hay input, an effect to which the Co supplementation appeared to contribute (Table 3.7). The subsequent expression of the parameters in terms of DM digested, produced significant ($p<0.05$) increases for total VFA and acetic acid output only (Table 3.6).

The fermentation in vessel 2 (periods 1, 2 and 3)

The atypical fermentation in vessel 2, after reinoculation, was demonstrated by both ADMD values (Fig. 3.3, Table 3.7) and the VFA molar proportions (Table 3.3). Values of ADMD were consistently lower than those of vessels 1, 3 and 4 until the latter stages of the experiment (period 3; Co supplementation); when they

TABLE 3.9 Experiment 1. Mean cobalamin outputs, expressed in three forms, for vessel 2 when 7 g DM low cobalt hay/d was given unsupplemented or supplemented with cobalt at two levels.

	Nominal cobalt supplement (nmol/d)		
	0	28.5	57.1
Period	1	2	3
a) pmol Cbl/d	356	342	341
b) pmol Cbl/d/g DM	50.9	48.9	48.7
c) pmol Cbl/d/g DM digested	93.0	79.7	81.8

aligned with the other vessels (Fig. 3.3). However, the abnormal proportion of valeric acid persisted (Table 3.8).

The high output and proportion of iso-valeric acid in the VFA were particularly noticeable when compared to the other vessels (Table 3.8). The Cbl output values for vessel 2 are shown in Table 3.9. The resting value was less than one-quarter those of the other vessels (Tables 3.4 and 3.9) and this was not increased by Co supplementation, even when ADMD increased to the level in the other vessels in period 3. During that period of the experiment when ADMD was depressed, pH values of the vessel liquid (compartment 1) were consistently 0.1-0.2 units lower than those of the other vessels, but this ceased with the increase in ADMD.

Discussion

Resting values with unsupplemented hay diet (period 1)

As a general guide to the fermentations established in Rusitec, ADMD might be considered the best indicator as it is the summation of many microbial activities. The 6 d period in Rusitec, taken by the microbial populations associated with the Ruminant A diet to adjust to digestion of a hay ration, was similar to that period reported for the equilibration in Rusitec when changing from a concentrate to a roughage diet, or vice versa (Czerkawski and Breckenridge, 1977). However, while 6 d may be suitable for stabilisation with regard to ADMD, the rate of change of other parameters may differ (Czerkawski and Breckenridge, 1977) and in future experiments it was considered necessary to study the equilibration of Cbl synthesis in more detail.

Once equilibrium was attained, the hay in Rusitec was digested to a slightly lesser extent than was anticipated from the *in vitro* cellulase method used by the Advisory Nutrition dept., East of Scotland College of Agriculture (0.604 and 0.643 respectively). However, VFA patterns were consistent with those found *in vivo* in animals on roughage diets.

The synthesis of Cbl from the Co in unsupplemented hay in vessels 1, 3 and 4 was apparently highly efficient when calculated from the data in Table 3.4. The mean efficiency of conversion, 39.6 ± 2.65 %, was in excess of previously reported *in vivo* determinations (Table 1.2). For a wheaten hay-chaff of comparable Co content (438 nmol/kg diet), Smith and Marston (1970a) calculated an efficiency of conversion of 11.4 %. However, other workers, using a predominately hay diet containing 798 nmol Co/kg diet, calculated an efficiency of 3.42 % (Hedrich *et al.*, 1973). The Ruminant A diet, which provided the inoculum, had a substantial Co content (>65 µmol/ kg diet DM), but at the time of sampling in Rusitec (d 48 and 50) any elevated vitamin B12 levels due to a carryover effect would be expected to have been diluted out. The possibility that the cultures were contaminated by Co from other sources must be considered.

Gawthorne (1970a) found Cbl levels in the strained rumen fluid of sheep fed a wheaten chaff diet of low Co content (679 nmol Co/kg diet) to be 64.2 nmol/l compared to mean levels of 2.61 nmol Cbl/l in compartment 1 of Rusitec. The assay of Gawthorne utilised strained rumen fluid and the

majority of both Cbl and analogues is considered to be associated with microbial populations of the solid phase (Smith and Marston, 1970a). However, the comparatively low level of Cbl in compartment 1 does not necessarily mean that Co contamination, as exemplified by the high conversion efficiencies, did not occur because Rusitec differs greatly from the *in vivo* system in having a high liquid: solid phase ratio and direct extrapolation of results from Rusitec may therefore be unwise. The physical differences between the *in vitro* and *in vivo* systems will be further discussed in Ch. 8 (p 214).

Effect of cobalt supplementation upon rumen fermentation (period 2)

The elevated levels of Cbl output upon supplementation with Co (Table 3.4) were consistent with previous reports (Hine and Dawbarn, 1954; Gawthorne, 1970a, 1970b; Hedrich *et al.*, 1973). The linear relationship with Co input (Fig. 3.4) agrees with the work of Gawthorne (1970a) who found a significant linear relationship ($r=0.976$, d.f.=3) between Co intake and Cbl levels *in vivo* for strained rumen fluid over the range 679-5,770 nmol Co/kg diet. Smith and Marston (1970a) found the conversion efficiencies of Co into Cbl to decrease linearly with increased dietary Co concentration to 3.05 % at supplemented intakes of 16.5 µmol Co/kg diet. This increasing efficiency with decreasing Co concentration is obviously a sound biological strategy. In Rusitec, contamination by Co would not affect the estimate of efficiency for inorganic Co derived from the linear regression. However, the efficiency value calculated for the unsupplemented hay (period 1) would be

The high levels of Cbl output in Rusitec raised the possibility of contamination. Assuming an overall conversion efficiency for Co into Cbl of 1.95 %, from the regression equation, the level of Co required to attain a resting value for Cbl output of 1505 pmol/d can be calculated as being 76.7 nmol Co/d, of which 3.8 nmol was supplied by the hay. Possible sources of contamination included the steel drive rods, each weighing 19 g, and the materials used in vessel construction. Of all these, the 4 steel drive rods are the most probable and the required loss of Co/rod would be 4.3 µg Co/d, which need not necessarily have affected the structure of the rods. Contamination by Co might also have been introduced by the artificial saliva. However, analysis of the artificial saliva for Co by the atomic absorption method (p 95) did not reveal any significant traces of the element.

There was an increase in the proportion of propionic acid in the total VFA and decreases in the total valeric acid proportion and the acetate: propionate ratio in the vessel fluid when Co was added. Reports of Co supplementation altering rumen fermentation, e.g. digestibility, VFA production (Young, 1979) have mostly referred to supplements well in excess of 85 µmol Co/kg diet. However, Saxena and Ranjhan (1978) increased digestibility, by Co supplementation at approximately 8.49 µmol/d, in calves fed "Co-adequate" rations. Uesaka et al. (1966a) found that Co added at 17.0 µmol/l to an in vitro system increased total VFA and propionic acid production by mixed rumen bacteria. The effect was dependent upon the carbohydrate source used,

Co being influential with starch and glucose but not with cellulose. A similar study of rumen protozoa showed no Co effect (Uesaka *et al.*, 1966b), although a Co requirement has been reported for an axenic, rumen protozoal culture (Durand and Kawashima, 1980). "Propionic acid producing" bacteria have been found to be significant producers of Cbl (Dryden *et al.*, 1962; National Institute for Research in Dairying, 1964) and the concomitant increases in propionate and Cbl output during Co supplementation may therefore have reflected the enhanced activity of these organisms.

Effect of an increased hay input upon fermentation during cobalt supplementation (period 3)

The effects of DM input (DMI) upon Cbl output *in vivo* have been inconsistent. Smith and Marston (1970a) found Cbl production to be limited by the DMI rather than Co when a Co-supplemented diet was pair-fed with a Co-deficient diet; but Hedrich *et al.* (1973), using a multiple regression technique, found no such effect. Increasing the hay input to Rusitec by 50 % reduced Cbl output at all 3 levels of Co supplementation. There was also a slight but significant decrease in ADMD which suggests that the system may have been overloaded, although significant increases occurred in total VFA and acetic acid output/g DM digested. Optimum levels of digestion in Rusitec are dependent upon the amount and type of food substrate and the highest levels of VFA or microbial production/g DM digested need not occur at the highest levels of digestibility (Czerkawski and Breckenridge, 1977). Izumi and Nishino (1974) showed molar proportions of VFA to alter with hay input *in vivo*; the proportion of butyric acid increased, while that of acetic and iso-valeric acids decreased with increasing hay input.

When the DMI was increased, 3 different mechanisms were likely to be operating in Rusitec.

1. Obstruction: Compartments 2 and 3 in Rusitec are the sites of microbial digestion and have a high concentration of microbes (Czerkawski and Breckenridge, 1979a). Increased feed input might restrict movement and/or colonisation of the microoganisms within these compartments, particularly with a less digestible ration, e.g. hay compared to concentrate rations. This is accentuated in Rusitec as the matter is retained in the feeding bag.

2. Dilution: The hay supplied comparatively little Co and the majority of the Co in this experiment was infused into compartment 1; the extent to which this became available to compartments 2 and 3 may have been reduced when DM inputs were increased and the surface area: volume ratio decreased. The 50 % increase in DMI enlarged the size of compartments 2 and 3, with a concomitant increase in microbial matter. Assuming that all the infused Co is available to this matrix, then the Co/unit microbial matter will decrease in response to an increase in matrix size. Increasing the hay input by 50 % reduced the Co input/unit hay DM by 33.3 %, e.g. from 2.04 to 1.36 µmol Co/kg hay DM, and Cbl output decreased similarly. Some nutrients appear to make this movement between compartments. It is noteworthy that urea infused into compartment 1 increased urease activity in all 3 compartments, although the percentage increases differed between compartments (Czerkawski and Beckenridge, 1982).

3. Sequestration: Rumen microbes are known to concentrate dietary Co, presumably with some as vitamin B12, at both deficient and adequate Co intakes (Tosic and Mitchell, 1948; Martinez, 1972). Czerkawski (1979) has defined compartment 2 as a "shuttle compartment" for sequestration of nutrients required at the site of digestion, compartment 3. The increased size of compartments 2 and 3 might lead to the disproportionately increased "trapping" of Cbl, as some species of bacteria have been found to store extremely high amounts of Cbl (Oginsky, 1952; Kashket et al., 1962). Therefore, by sampling compartment 1, output values might underestimate Cbl synthesis unless the system has equilibrated.

The fermentation in vessel 2 (periods 1, 2 and 3)

Upon reinoculating vessel 2, from an animal fed upon Ruminant A diet, the fermentation was expected to be comparable to that achieved previously; but the results for ADMD and VFA suggested otherwise (Fig. 3.3, Table 3.3). Although the fermentation was predominately "acetic", the low acetate: propionate ratio was more representative of a ration containing a substantial proportion of concentrate. The most noticable difference was the increased molar proportion of total valeric acid. This was due to an elevated output of iso-valeric acid (Table 3.8), which can be synthesised by deamination and decarboxylation of leucine or iso-leucine (McDonald et al., 1977). If 10 % of the crude protein of the hay is considered to be leucine or iso-leucine (McDonald et al., 1977), then this would account for less than 20 % of the daily output of total

valeric acid. The source of the extra valeric acid was unknown, but Czerkawski and Breckenridge (1979a) found an increased production of iso-valeric acid and a reduced digestibility of hemicellulose when N was limiting in a Rusitec culture given a hay ration and the microbial matter in that culture had high levels of carbohydrate. Possibly a deranged fermentation of carbohydrate in vessel 2 produced the unusual levels of iso-valeric acid. Cline et al. (1958) had also found the levels of valeric acid to increase when rumen microbes were grown in a N-deficient medium and they implicated reduced valeric acid levels in increased cellulytic activity and microbial growth.

The in vitro fermentation of 1,2-propanediol, a propiogenic food additive, by rumen microbes has been found to produce a characteristic odour (Czerkawski and Breckenridge, 1972) and this fermentation pathway was later shown to produce elevated amounts of propionic and 2-methyl butyric (a valeric acid isomer) acids, in addition to depressing digestibility by 5-12 % as the level of 1,2-propanediol added was increased (J. Czerkawski, personal communication, 1982). In Experiment 1, the culture in vessel 2 was found to produce a "sweet" odour that distinguished it from the other cultures and that was not unlike the odour of the iso-valeric mixture used as a VFA standard (p 93) or that of 2-methyl butyric acid; in addition, digestibility of the ration was decreased by 7 %. Therefore, increased production of 2-methyl butyric acid might have accounted for the higher levels of iso-valeric acid in this experiment, even though the hay ration was the same for all 4 cultures.

Although the occurrence of the atypical fermentation in vessel 2 was fortuitous, the ability to instigate such a fermentation might be of considerable practical significance as the molar proportion of propionic acid was increased considerably. However, the effects upon other aspects of the fermentation, such as microbial growth and the host's Cbl requirements, would have to be investigated.

Prior to the leak appearing in vessel 2 there were no marked differences in performance between the vessels (Fig. 3.2). Therefore, differences were not due to vessel construction. There are 3 possible reasons for the unusual fermentation which occurred.

1. It is possible that in permanently sealing the vessel, compounds from the epoxy resin sealant leached into the vessel and were either toxic to certain microbes or were fermented themselves. Such effects might be expected to decrease with time as the active compounds were removed by the effluent or the microbial populations adapted to the toxin. The high total valeric acid proportion, however, persisted throughout the experiment although the depressed ADMD did not.

2. Sampling rumen contents via a rumen fistula might have produced a different microbial population for the inoculation of vessel 2. Other workers have stressed the necessity of obtaining inocula representative of the heterogenous contents in the rumen for _in vitro_ studies (Senshu _et al._, 1980). However, the large volume of rumen contents (500 ml strained rumen fluid, 80 g strained rumen

solid) required to initiate each Rusitec culture would be expected to minimise this problem.

3. Different donor animals might have different rumen fermentations, when fed the same diet, which would be transmitted to an *in vitro* system. Hoover *et al.* (1976) fed donor animals the same diet in equal quantities in order to avoid differences between inocula for *in vitro* cultures, which they suspected had been responsible for variation between fermentations. Inter-animal differences could be related to the phenomenon reported by Czerkawski (1980) when the fermentation of glucose or sucrose was altered both within individual sheep and in concurrent *in vitro* experiments, for no apparent reason. It is customary for the inocula for Rusitec to be obtained using rumen fistulae, preferably from animals fed the same ration to that proposed for the study.

Unfortunately, no firm conclusions could be reached regarding the source of the unusual fermentation in vessel 2.

The low level of Cbl output associated with Co supplementation of vessel 2, which persisted even when the ADMD increased, was unexpected and there appear to be no similar reports in the literature. The VFA patterns were characteristic of a "concentrate fermentation"; in which case, Cbl biosynthesis might be expected to be less than that for a "roughage fermentation" of comparable Co input (Sutton and Elliot, 1972). However, other possibilities have to be considered.

1. The growth of Cbl-synthesising bacteria may have been suppressed by non Cbl-synthesising microbes or their

metabolites. The growth of some rumen bacteria is known to be depressed by the addition of certain VFA salts (Stewart, 1975). The high levels of iso-valeric acid may have similarly depressed growth of Cbl-synthesising bacteria in vessel 2.

2. Synthesis of Cbl may have been normal but excessive microbial degradation of Cbl might have occurred (Smith and Marston, 1970a). This might have arisen if Cbl was inadequately protected by binding as a result of the unusual fermentation.

3. Co in the vessel may have been made unavailable, e.g. by the epoxy resin acting as a cation exchanger.

Low levels of Cbl may, themselves, have been responsible for the abnormal VFA proportions. Again, there are a number of possibilities to be considered.

1. The low level of Cbl restricted utilisation of propionate by the microbes. Consequently, propionate accumulated and some may have been converted to valeric acid (Van Campen and Matrone, 1960).

2. Some bacterial species are known to synthesise acetic acid using a Cbl-dependent pathway (Table 1.4). Therefore, the low levels of Cbl might have been responsible for the lowered acetic acid output.

3. In mammals, iso-leucine is metabolised via propionate. If a comparable pathway exists in microorganisms then this Cbl-dependent catabolism would not proceed and iso-leucine may have been converted into iso-valeric acid.

It would be interesting to know whether analogues of Cbl had also been synthesised in smaller quantities, as many microbial pathways can function with forms of vitamin B12 other than Cbl (Babior, 1975; Poston and Stadtman, 1975).

Conclusions

A number of important conclusions can be drawn from this experiment.

1. Variation in fermentation between vessels may have occurred as a result of the inoculation procedure.
2. For 3 vessels given the same inoculum, VFA patterns and ADMD were uniform and typical of roughage diets; but there were large differences in Cbl output. A fourth vessel which had to be reinoculated developed an abnormal fermentation with low acetic, high propionic and valeric acid molar proportions and a low Cbl output.
3. A low efficiency of incorporation of inorganic Co into Cbl (1.95 %) raised the possibility of differential distribution of nutrients between compartments and it highlighted possible differences between *in vivo* and *in vitro* experiments.
4. An apparently high efficiency of incorporation of Co from the low Co hay into Cbl (39.6 %) suggested that some degree of Co contamination may have occurred and precautions were taken in further studies in view of the low levels of contamination required to elicit a response in vitamin B12 synthesis.
5. Increasing DMI was found to reduce Cbl output and consequently the lower level (7 g DM/d) was employed in Experiment 2.

CHAPTER 4

EXPERIMENT 2a. THE INFLUENCE OF INDIVIDUAL DIFFERENCES IN FERMENTATION UPON THE RUMINAL SYNTHESIS OF VITAMIN B12

Introduction

The atypical fermentation encountered in Experiment 1 was associated with a lowered synthesis of vitamin B12 and it was not influenced by additions of Co well in excess of the minimum nutrient requirements (ARC, 1980). It is possible that individual differences in the rumen microbial populations of the 2 donor sheep were maintained in Rusitec in Experiment 1. Alternatively, enviromental factors may have been involved. Periods of unstable fermentation, when the production of methane decreased, have been found concurently, in sheep and in *in vitro* fermentation systems (Czerkawski, 1980). Therefore, an attempt was made to determine whether observed differences in total vitamin B12 concentrations in the rumen could be maintained in Rusitec and if they were associated with particular fermentation patterns.

Experimental procedure

Samples of rumen contents were taken via fistulae from a group of 11 Scottish Blackface sheep given the Ruminant A diet, immediately before and 4 h after feeding, on 2 separate days. These samples were centrifuged at 2000 g for 15 m and the supernatant analysed for VFA and also total vitamin B12, using the nonspecific c.p.b. method (p 83). The ranking of these samples, according to the level of total vitamin B12, was consistent between sampling occasions.

TABLE 4.1 Experiment 2. Experimental design for Rusitec vessels 1-4, in a two part experiment, a) to study the effects of inoculum source on fermentation and b) to compare the vitamin B12 synthesis from three cobalt-adequate hays compared with that from inorganic Co. The food substrate was 7 g DM low cobalt hay(1) /d until period 2 of Experiment 2b.

	Vessel 1	2	3	4
Experiment 2a				
Donor sheep number*	1	9	6	8
Cbl in inocula	high	high	low	low
Experiment 2b				
Period 1				
+ Co (nmol/d)	<---47.5--->		<---23.8--->	
Period 2				
Substrate	low Co hay(1)	Augusta ryegrass early	late	Astra clover

*= see Table 4.2 for ranking

As mentioned on p 81, Rusitec was cleaned thoroughly before assembly, using a laboratory detergent, in order to reduce the possibility of Co contamination. Two pairs of donor animals were selected from the group of 11 sheep, 1 pair with a high total vitamin B12 level, the other pair with a low value. Inocula for the 4 separate vessels of Rusitec were obtained from these animals on a fifth occasion, 4h after feeding. The pair of inocula with "high" Cbl levels were used to instigate fermentations in vessels 1 and 2, the "low" Cbl inocula being introduced into vessels 3 and 4. The feed input to Rusitec was 7 g DM/d of the low Co hay(1), used in Experiment 1 (Table 3.1). The design for the whole of Experiment 2 is outlined in Table 4.1. Samples for analysis were taken regularly from the vessels for 12 d, prior to feeding. These were analysed for Cbl, VFA and, on specific dates, for total vitamin B12 (L.leichmannii method, p 91). Close attention was paid to both ADMD and AOMD as indicators of the stability of the fermentations. On d 12, samples were taken from the vessels at regular intervals throughout 24 h and analysed for Cbl. The dilution rate was maintained at 0.75 /d throughout Experiment 2.

TABLE 4.2 Experiment 2a. Levels of vitamin B12 (nmol/l), measured by the nonspecific, radioisotopic dilution assay, in clarified rumen liquid from sheep sampled, immediately before (0900) and 4 h after (1400) feeding, on 2 separate days.

	Day							
	4-11-80			6-11-80				
Sampling time	0900h	1400h	mean	0900h	1400h	mean		
Sheep number							Overall mean	ranking
1	29.0	31.2	30.1	16.7	9.96	13.3	21.7	10*
2	47.2	39.5	43.4	18.6	9.15	13.9	28.7	3=
3	35.1	42.0	38.6	17.3	10.3	13.8	26.2	7
4	36.9	50.4	43.7	18.3	9.23	13.8	28.7	5=
5	39.9	36.5	38.2	15.4	8.27	11.8	25.0	8=
6	42.4	50.1	46.3	26.4	10.3	18.4	32.3	1*
7	32.0	34.0	33.0	24.1	10.4	17.3	25.1	5=
8	43.5	28.7	36.1	19.9	10.9	15.4	25.8	3=*
9	29.2	26.1	27.7	17.6	6.43	12.0	19.8	11*
10	29.5	24.7	27.1	17.5	13.7	15.6	21.4	8=
11	35.9	35.1	35.5	21.3	15.1	18.2	26.9	2

* = animals selected as donors

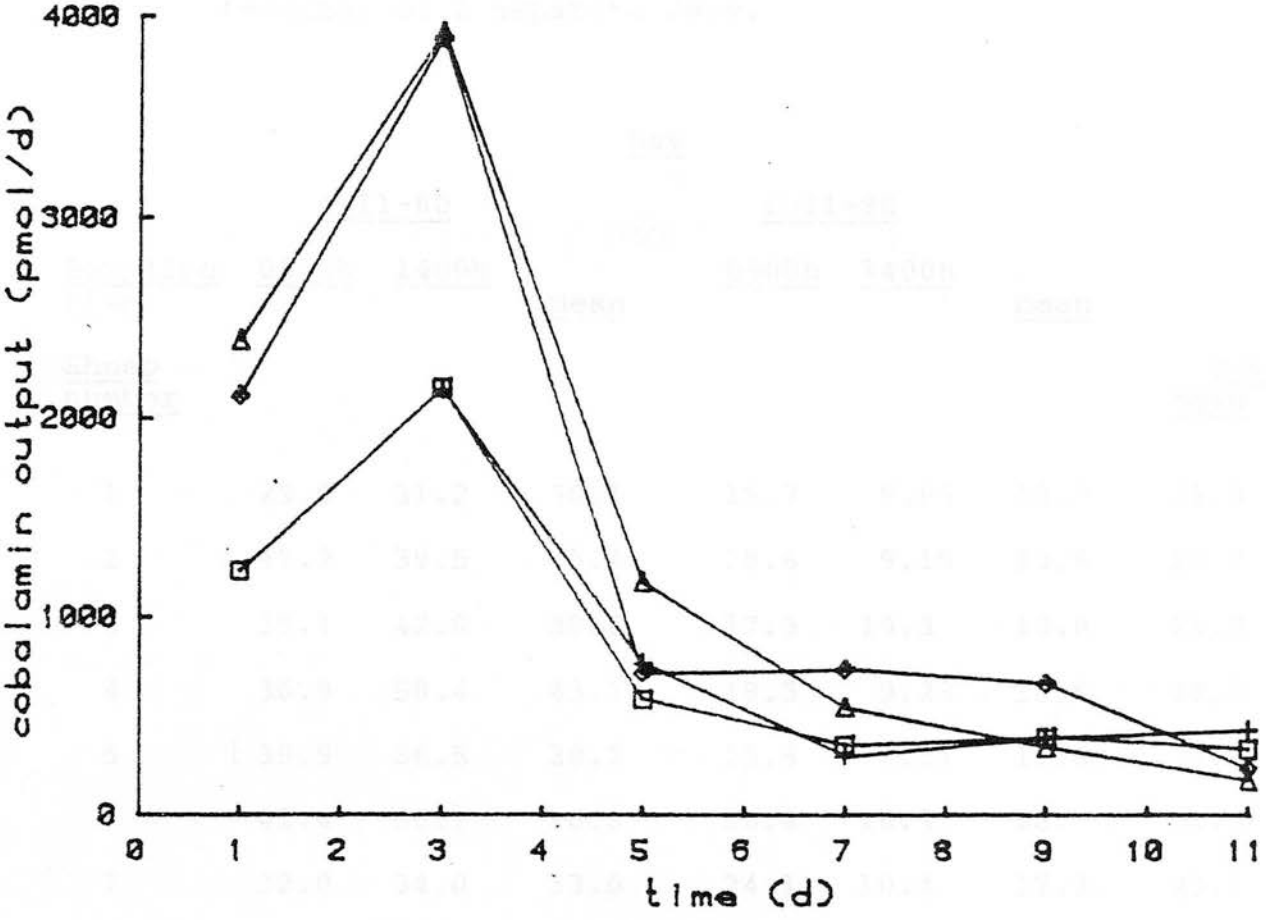


FIG. 4.1 Experiment 2a. Cobalamin output from vessels 1-4 given low cobalt hay(1) at 7 g DM/d following inoculation with rumen contents of high (vessels: 1= +, 2= △) and low (3= □, 4= ◇) Cbl contents.

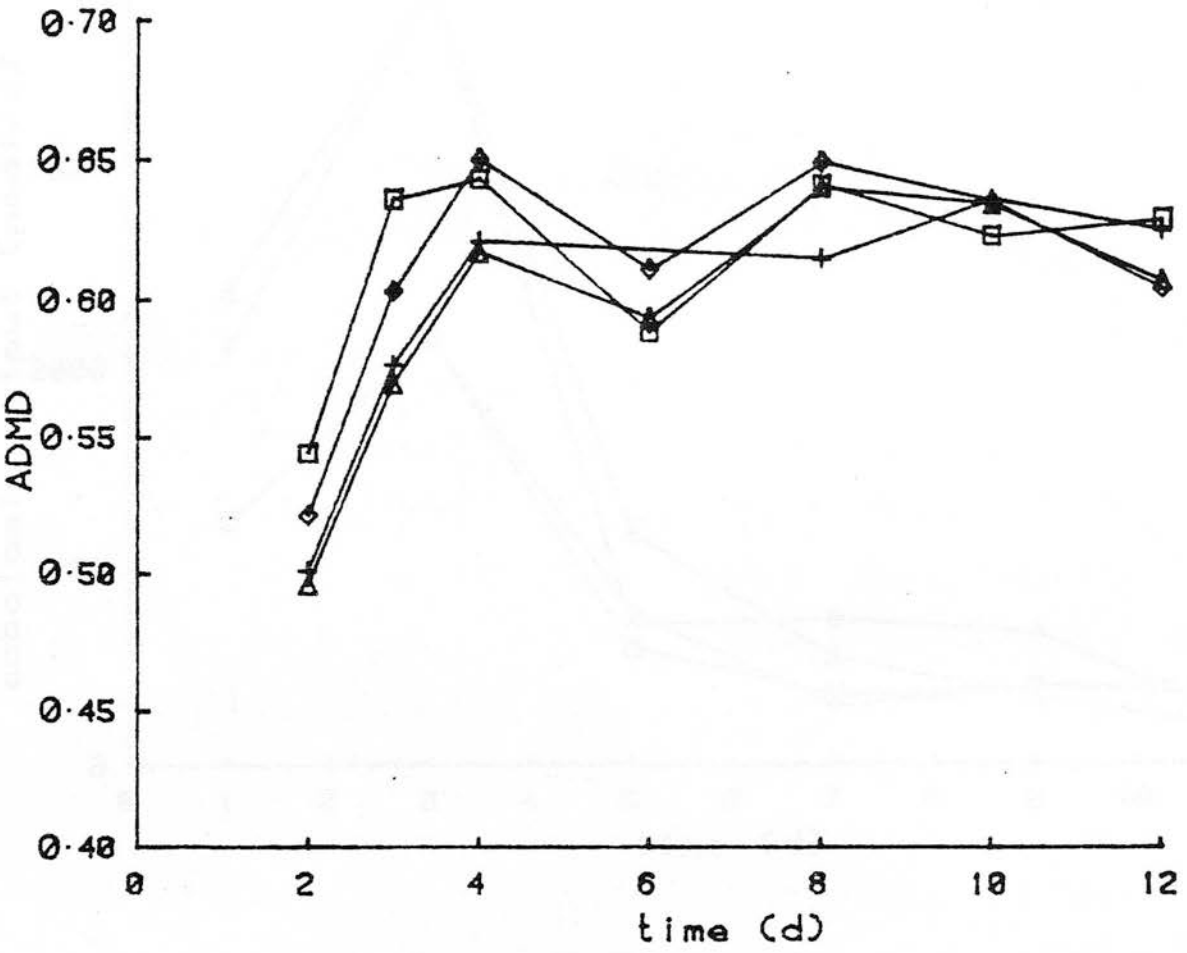


FIG. 4.2 Experiment 2a. ADMD of low cobalt hay(1) given to vessels 1-4 at 7 g DM/d.

(vessels: 1= +, 2= △, 3= □, 4= ◇)

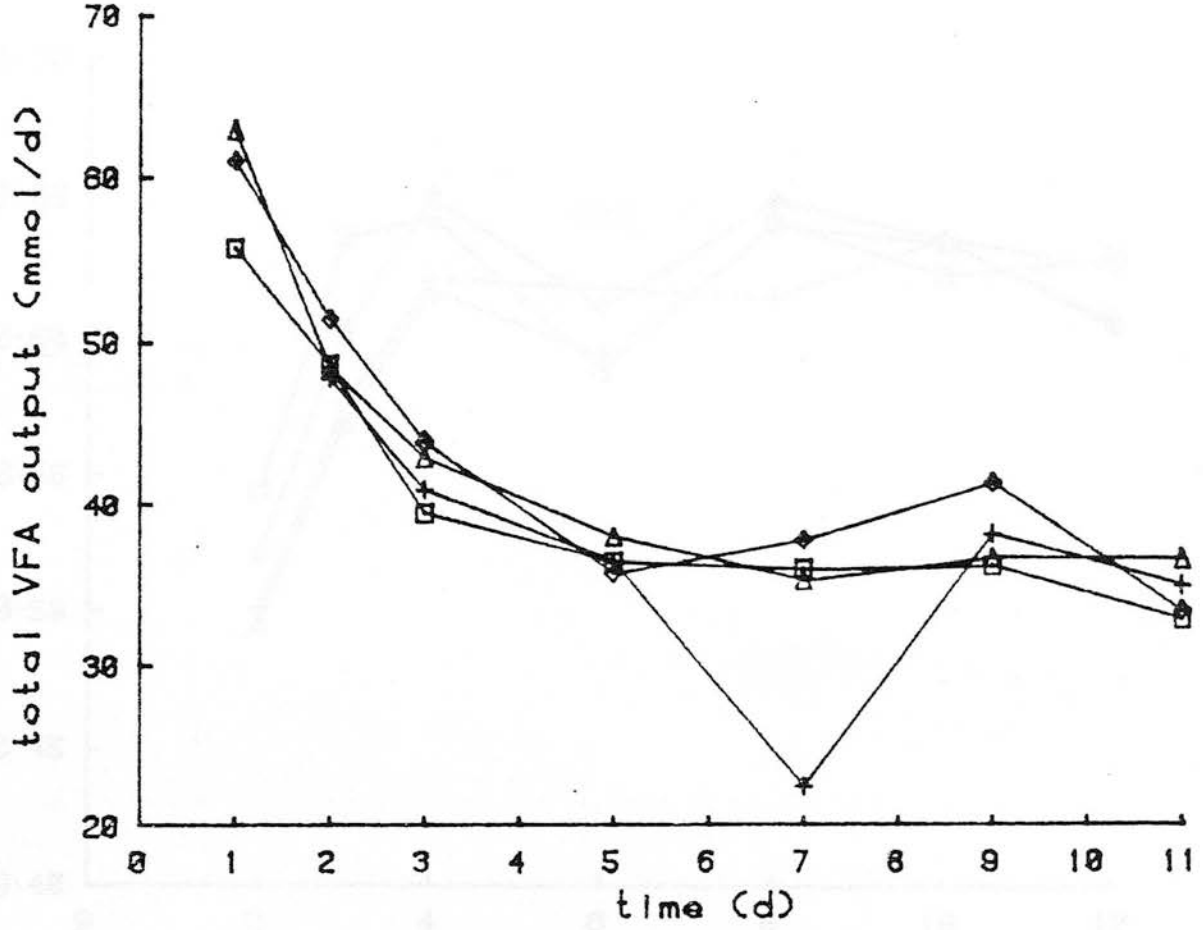


FIG. 4.3 Experiment 2a. Total VFA output from vessels 1-4 given low cobalt hay(1) at 7 g DM/d.
(vessels: 1= +, 2= △, 3= □, 4= ◇)

TABLE 4.3 Experiment 2a. Values for cobalamin (mean of d 7, 9 and 11) and analogue (d 11) output (pmol/d), analogue: cobalamin ratio and conversion efficiencies (%) for vessels 1-4. Sample from vessel 4 for analogue assay was lost.

	Vessel				
	1	2	3	4	mean ± s.d.
Output					
Cobalamin	374	353	361	545	408 ± 91.6
Analogue	4820	1330	1800	-	2650 ± 1890
Analogue: cobalamin	12.9	3.77	4.99	-	7.22 ± 4.96
Conversion efficiency					
Cobalamin	9.84	9.29	9.50	14.3	10.7 ± 2.39
Analogue	127	35.0	47.4	-	69.8 ± 49.9

Results

Inocula differences and subsequent resting values

The values for total vitamin B12 (Table 4.2) for the 11 animals sampled as possible donors were examined using analysis of variance. This treated each sheep as a separate group and assumed no sheep x day or sheep x time interactions. Differences between sheep were just significant ($p=0.05$) but there was a significant ($p<0.001$) difference between mean values for the 2 days and a significant ($p<0.01$) time of feeding x day interactions. Values declined significantly ($p<0.01$) after feeding on the second day (means, 19.4 vs. 10.3), but not on the first (means, 36.4 vs. 36.2). The highest levels on any occasion ranged from 1.6-2.3 times those of the lowest levels (Table 4.2).

Variation in Cbl output between vessels was marked. The inter-vessel c.v. for Cbl output was 30-40 % for all Cbl measurements up to d 11 (App. 2.1), compared to typical values for ADMD of less than 5 % and for VFA of less than 10 %. The differences were not, however, attributable to the use of high and low Cbl inocula. Although vessels 1 and 2 had inocula from donors with an approximately 50 % higher concentration of total vitamin B12 (as measured by the nonspecific c.p.b. method) than that given to vessels 3 and 4, differences between vessels, due to inocula, did not occur (Figs. 4.1, 4.2, 4.3). Minimum Cbl and analogue output values were achieved by d 7 and d 11 respectively. Mean Cbl values for this period were calculated from the figures for d 7-11 (n=3), while analogue values were those for d 11 only, for which the sample for vessel 4 was lost (Table 4.3).

TABLE 4.4 Experiment 2a. VFA parameters (mean ± s.d.) for four cultures given low cobalt hay(1) at 7 g DM/d and different inocula and sampled on d 7, 9 and 11.

	Mean ± s.d. (n=4)
VFA proportions (%)	
Acetic acid	64.4 ± 0.881
Propionic acid	19.9 ± 1.09
Total butyric acid	10.4 ± 1.04
Total valeric acid	5.34 ± 0.764
Acetate: propionate	3.25 ± 0.154
VFA output (mmol/d)	
Total VFA	35.1 ± 2.40
Acetic acid	22.6 ± 1.48
Propionic acid	6.96 ± 0.603
Total butyric acid	3.63 ± 0.468
Total valeric acid	1.88 ± 0.321

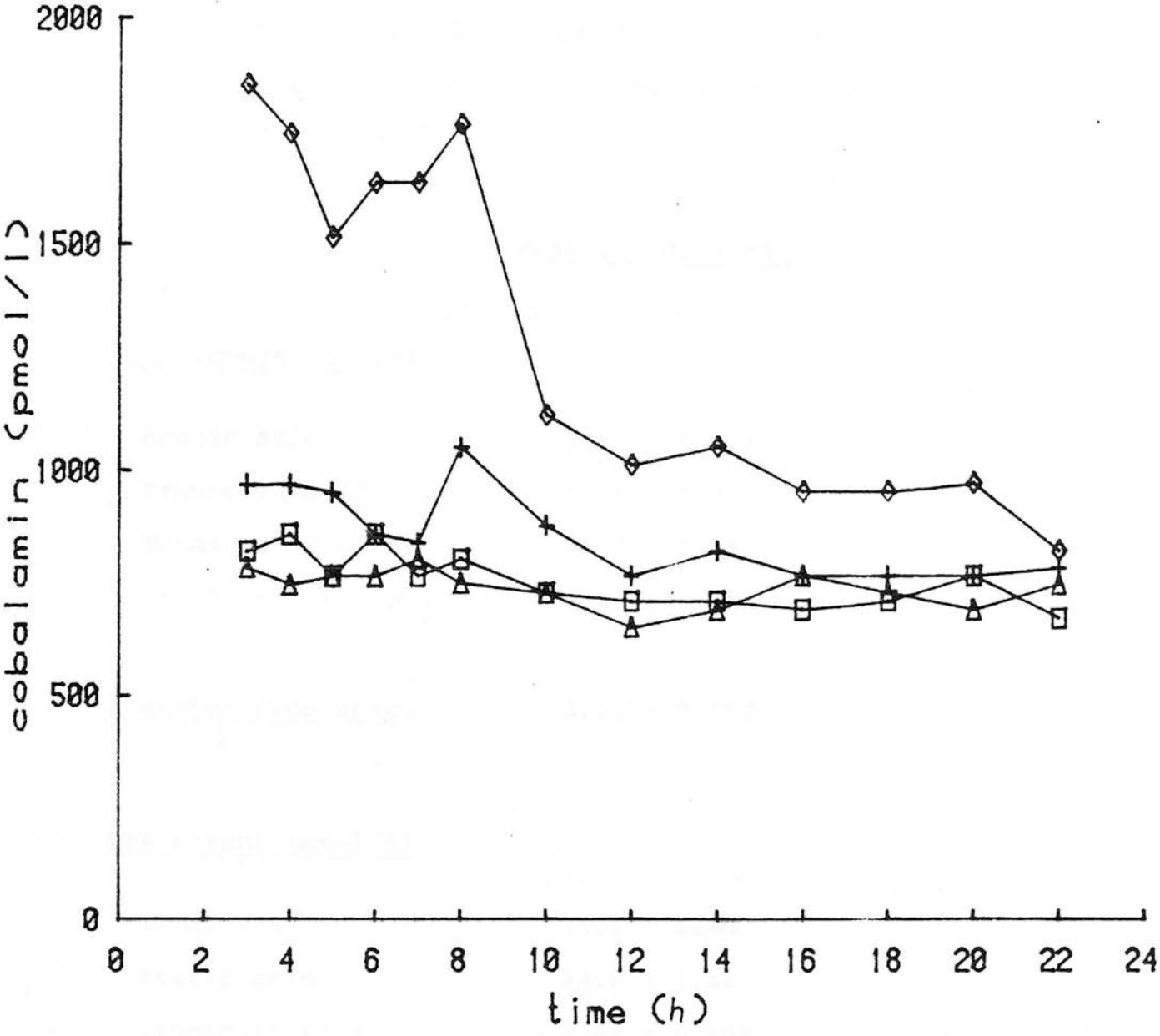


FIG. 4.4 Experiment 2a. Diurnal variation in cobalamin levels in compartment 1 of vessels 1-4, given low cobalt hay(1) at 7 g DM/d on d 12. (vessels: 1= + , 2= △ , 3= □ , 4= ◇)

The resting values of Cbl output for vessels 1-4 had a c.v. of 22.5 %, but the value for vessel 4 was noticeably higher than those for the other vessels. Variation in analogue output in vessels 1-3 was greater than that for Cbl output (c.v.=71.3 %). Conversion efficiencies for both Cbl and analogue synthesis were calculated (Table 4.3). For Cbl these efficiencies covered the range 9.29-14.3 % and for analogue synthesis the range was 35.0-127 %.

VFA outputs declined until the 5th day but throughout this period were similar for all 4 vessels and characteristic of a roughage fermentation (Fig. 4.3, Table 4.4). Digestibility values reached a maximum on d 4 (Fig. 4.2), were similar in all vessels (c.v. <1 %) and only 2 % less than the predicted in vitro cellulase values (Table 3.1).

Diurnal variation of cobalamin levels in compartment 1 The data for Cbl levels throughout 24 h (Fig. 4.4) confirmed the different behaviour of vesssel 4. In this vessel there was a marked decrease in the Cbl concentration of compartment 1, over the period 3-10 h, and a slower decline for the remaining 12 h. Cbl levels were higher than those in vessels 1-3, which varied little throughout the 24 h.

Estimates of the Cbl content of the reintroduced washings (compartment 2) were made from the values obtained throughout 24 h. A basal or pre-feeding Cbl level was obtained by extrapolation of the stable values to 0 h. From the difference between this value and that of the post-feeding Cbl level plus the volumes of the washings and of compartment 1, 110 and 550 ml respectively,

determinations were made of the Cbl content of the washings. The calculations gave values of 317, 282, 254 and 946 pmol Cbl for vessels 1-4 respectively. Estimates of the Cbl production for d 12 were made by determining the area under the Cbl vs. time curve (area method) and this gave values of 851, 735, 746 and 1260 pmol for vessels 1-4 respectively.

Discussion

Differences between potential donor animals

No correlations were found between levels of total vitamin B12 and any of the VFA parameters in the rumen fluid samples from the 11 potential, donor animals. While ranking for Cbl was maintained on the 4 sampling occasions there were large variations both between days and between sampling times on any one day. Inter-day variation may have been large because the Ruminant A ration was increased from 1-1.2 kg diet/animal/d between sampling days and had been given for just 6 d prior to the first sampling. However, Gawthorne (1970a) also found substantial variation in the total vitamin B12 level of rumen fluid when sampling single animals over 8 months. A similar effect of time after sampling has been reported by Smith and Marston (1970a), who found that levels of Cbl in rumen fluid dropped after feeding to a minimum after 4 h.

Inocula differences and subsequent resting values

The "high" and "low" vitamin B12 inocula did not instigate different Cbl outputs or fermentations; therefore, it seems unlikely that differences between vessels, seen in the

first experiment, resulted from the carryover of differences between donor sheep. Vessel differences were, however, again a feature of the results (Fig. 4.1, Table 4.3) and it was surprising to find that the high Cbl output for vessel 4 originated from a "low" inoculum.

The Cbl outputs and ADMD achieved equilibrium within 5-7 d (Figs. 4.1, 4.2), but the time taken for vitamin B12 analogue output to stabilise was longer than 7 d (App. 2.1). This contrasts with the data of Tressol and Lamand (1979) who found that the minimum level of total vitamin B12 *in vivo* (as measured by *L.leichmannii* ATCC 7830) was achieved within 7 d after withdrawing a Co supplement from a Co-deficient hay diet. Rusitec retains the solid fraction of the diet for 48 h and with it the sequestered microbial matter (Czerkawski, 1979). The retention of the vitamin B12-rich, microbial matter for longer than would occur *in vivo*, could delay the attainment of equilibrium for both Cbl and analogue output. Furthermore, the delay may be different for different forms of the vitamin. Both *E.coli* (Oginsky, 1952) and *L.leichmannii* (Kashket *et al.*, 1962) can accumulate Cbl well in excess of their growth requirements. *L.leichmannii* was considered to be capable of storing 9000 times its minimum growth requirement and uptake was shown to increase with an increasing concentration of Cbl in the media. Similar effects may also occur with the metabolically active analogues. The influx of large amounts of the vitamin in the inoculum might have allowed uptake of both the free and bound vitamin by microbes, as this has been shown to occur with Cbl for both aerobic (Giannella *et al.*, 1972) and anaerobic (Welkos *et al.*, 1981) bacteria.

The reduction of VFA output in vessel 1 on d 7 was due to less nutrient being available for fermentation because of the removal of the wrong bag on d 6. However, this reduced fermentation did not significantly affect Cbl output and again there is the possibility that Cbl outputs were "buffered" by the storage capacity of the microbes.

The conversion efficiencies of Co into Cbl (Table 4.3) were approximately one quarter of those for the same hay in Experiment 1 and were in general agreement with values calculated by Smith and Marston (1970a) for a Co-deficient hay (11.4 %). This suggests that Co contamination might have occurred in Experiment 1 and had been reduced by thoroughly soaking the apparatus in a detergent prior to assembly. Efficiencies of conversion for Co into analogues ranged from 35.0-127 %. However, it appeared that equilibrium was not achieved in vessel 1, and possibly vessel 4, by d 11. Values for analogue output were therefore likely to be overestimates of daily output and it may explain why the ratio of analogue: Cbl for the fermentation in vessel 1, even on d 11, was much greater (11.1) than those reported for Co-depleted and Co-repleted animals, 7.22 and 3.00 respectively (Smith and Marston, 1970a). However, the difference may be due to the different analytical methods used for Cbl determination; i.e. P.malhamensis vs. the c.p.b. radioassay used in this study.

The peak values for VFA (Fig. 4.3) and Cbl (Fig. 4.1) were attained after 1 and 3 d respectively. The early peak in VFA values was considered to be due to increased microbial

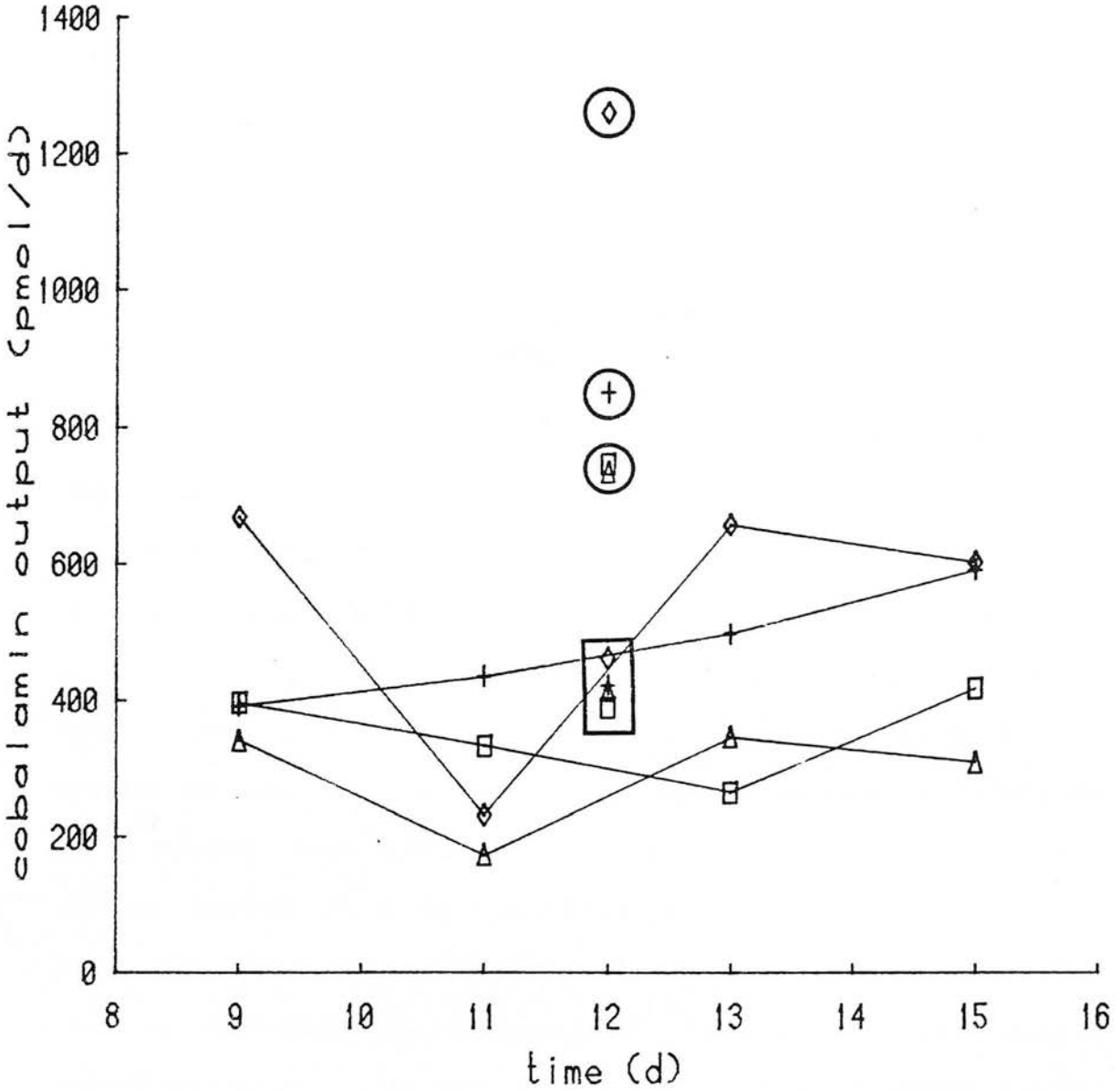


FIG. 4.5 Experiments 2a and 2b. Cobalamin output values from vessels 1-4 on d 9, 11, 13 and 15, compared with productivity values on d 12 calculated from the diurnal samples by;
a) the area method (encased in circle),
b) extrapolation of the stable diurnal values to 24 h and determination of an output value (encased in square).

activity during the first 24 h, e.g. colonisation of fresh substrate, alteration in microbial populations. The later peak for Cbl, suggested that there was a link between Cbl synthesis and fermentation activity which, although it may occur concurrently in the microbe, was not manifest in Cbl output values for approximately 48 h. The recycling of the different forms of the vitamin, mentioned previously (p 120), may also have contributed to this "lag" effect. Recycling of Cbl would have been greatest in compartments 2 and 3 (the feed matrix) because of their physical restriction in the feed bags and the inclusion of the nutrient-rich inocula.

The decline in output values for VFA and Cbl after d 1 and d 3 respectively, was due to the reduced amount of substrates provided by the low Co hay(1).

Diurnal variation of cobalamin levels Cbl outputs would be underestimated by sampling compartment 1 at 24 h if there were elevated values in the period after feeding, as occurred with vessel 4. The estimate of Cbl production from vessel 4 on d 12, derived from the area under the Cbl vs. time curve, was 5.5 times greater than the estimated output for this vessel on d 11 (Fig. 4.5) and 1.9- 4.2 times higher than for the other vessels. The difference between these values might be greater because a higher rate of gas formation in the immediate post-feeding period may have increased outflow by displacing fluid from compartment 1 when Cbl concentrations were high.

Using a final diurnal value, derived by extrapolation to 24 h of the stable diurnal values, an estimate can be made for Cbl output on d 12. The agreement between these values and those from days when diurnal sampling was not undertaken was good and is shown in Fig. 4.5.

The high, initial Cbl levels in vessel 4 were assumed to be due to the reintroduction of Cbl-rich washings. The provisional estimates of the Cbl content of the washings (254-946 pmol) were of the same order as daily outputs. That compartment 2 can be so influential is possibly due to it containing a significant proportion of the microbial population in Rusitec (31 % of the bacteria and 56 % of the protozoa; Czerkawski and Breckenridge, 1979a) in close proximity to the nutrient source. These microbes are probably the major synthesisers of both Cbl and analogues and the considerable amounts of Cbl in compartment 2 support earlier suggestions that it could have a marked "buffering" capacity. Furthermore, the reintroduced washings were from the more completely digested feed (48 h) and the Cbl content of compartment 2 from the partially (24 h) digested feed may have been even greater. Confirmation of the role of compartment 2 was sought in later experiments by sampling the washings for Cbl content.

Some decline in Cbl concentrations in compartment 1 after feeding was to be expected simply as a result of dilution by the artificial saliva. However, the dilution rate would only be 4.5 %/h, assuming a compartment 1 volume of 550 ml and an artificial saliva input of 600 ml/d. Therefore, it seems likely that there is sequestration of Cbl by the

other compartments. Colonisation of a new food substrate occurs in 2 phases (Mehra et al., 1981); there is an initial colonisation of compartment 2 and the digestion of non-fibrous material (0-10 h), followed by colonisation of compartment 3 and fibre digestion. Such processes will cause compartment sizes to vary with time (Czerkawski, 1979), as do enzyme activities in compartment 2 (Brice and Morrison, 1981). Thus, sequestration of microbes by the feed matrix would be expected to account for a significant proportion of the drop in Cbl levels in compartment 1. In addition, the sequestered bacteria may be capable of adsorbing some free or non-microbially bound Cbl from compartment 1 (Giannella et al., 1972; Welkos et al., 1981).

In vessels 1-3 the absence of high Cbl levels at 3 h suggested a much lower Cbl content in the washings, though still sufficient for normal, microbial metabolism. With smaller amounts of Cbl being reintroduced it is not surprising that stable levels were likely to be achieved more rapidly. Stability will be achieved when the increase of Cbl in compartment 1, from synthesis and flow from compartment 2, is equivalent to the flow into compartment 2 plus the the loss in the vessel effluent. These low levels of Cbl may also have influenced the quantity of Cbl available for analysis. At lower concentrations, fewer Cbl molecules are likely to be retained on the cell wall as the absorption process will not be saturated (Sennett et al., 1981) and once absorbed these molecules are probably not available for analysis (p 87).

The unusual diurnal Cbl pattern of vessel 4 (Fig. 4.4) during d 12 was unexpected in view of the uniformity of Cbl output upto that time and the low output value for vessel 4 on the preceding day (Fig. 4.5). Daily variation in diurnal rhythms may therefore occur even when a culture has apparently stabilised.

Conclusions

1. The synthesis of Cbl in cultures derived from 4 different inocula was not related to the level of Cbl in the inoculum nor were any differences revealed between cultures in VFA output or ADMD.

2. Studies of diurnal fluctuations in Cbl concentrations in vessel fluid, revealed high values immediately after feeding following the redistribution of washings (compartment 2). This drew attention to the important role of compartment 2 in Cbl synthesis and as a contributor to vessel differences in Cbl output. Furthermore, sampling from within the vessel at 24 h would estimate "resting output", but underestimate total Cbl production (Ch. 8, p 208). Unfortunately, these implications were not recognised in time for procedural changes to be instigated in Experiment 2b.

EXPERIMENT 2b. AN INVESTIGATION OF VITAMIN B12 SYNTHESIS FROM DIFFERENT ROUGHAGE DIETS

Introduction

High roughage diets are reported to increase the proportion of Cbl in total vitamin B12 produced in the rumen compared to concentrate diets (Sutton and Elliot, 1972). Having shown no significant inocula differences for either Cbl output, VFA output or ADMD in Experiment 2a, the same cultures were used in an examination of the effect of different hays on vitamin B12 synthesis. As the hays differed in Co content, a comparison was also made with inorganic Co supplements added to the low Co hay(1).

Experimental procedure

Experiment 1 aroused concern over the possibility of contamination by Co in the Rusitec vessels. Therefore, the investigation of responses to Co supplements was repeated in this experiment and the treatments were duplicated in order to assess inter-vessel variation before assessing vitamin B12 synthesis from different hays.

In period 1, 47.5 nmol Co/d (0.40 mg Co/kg DM) was infused with the artificial saliva into vessels 1 and 2 for 13 d (d 13-25), while vessels 3 and 4 received 23.8 nmol Co/d (0.20 mg Co/kg DM). Supplementation of the low Co hay(1) was discontinued for 3 d (d 26-28) to permit some depletion of Co levels before a comparison of different hays was attempted. In period 2 (d 29-40) the low Co hay(1) was used in vessel 1 and hays of Augusta ryegrass (early or

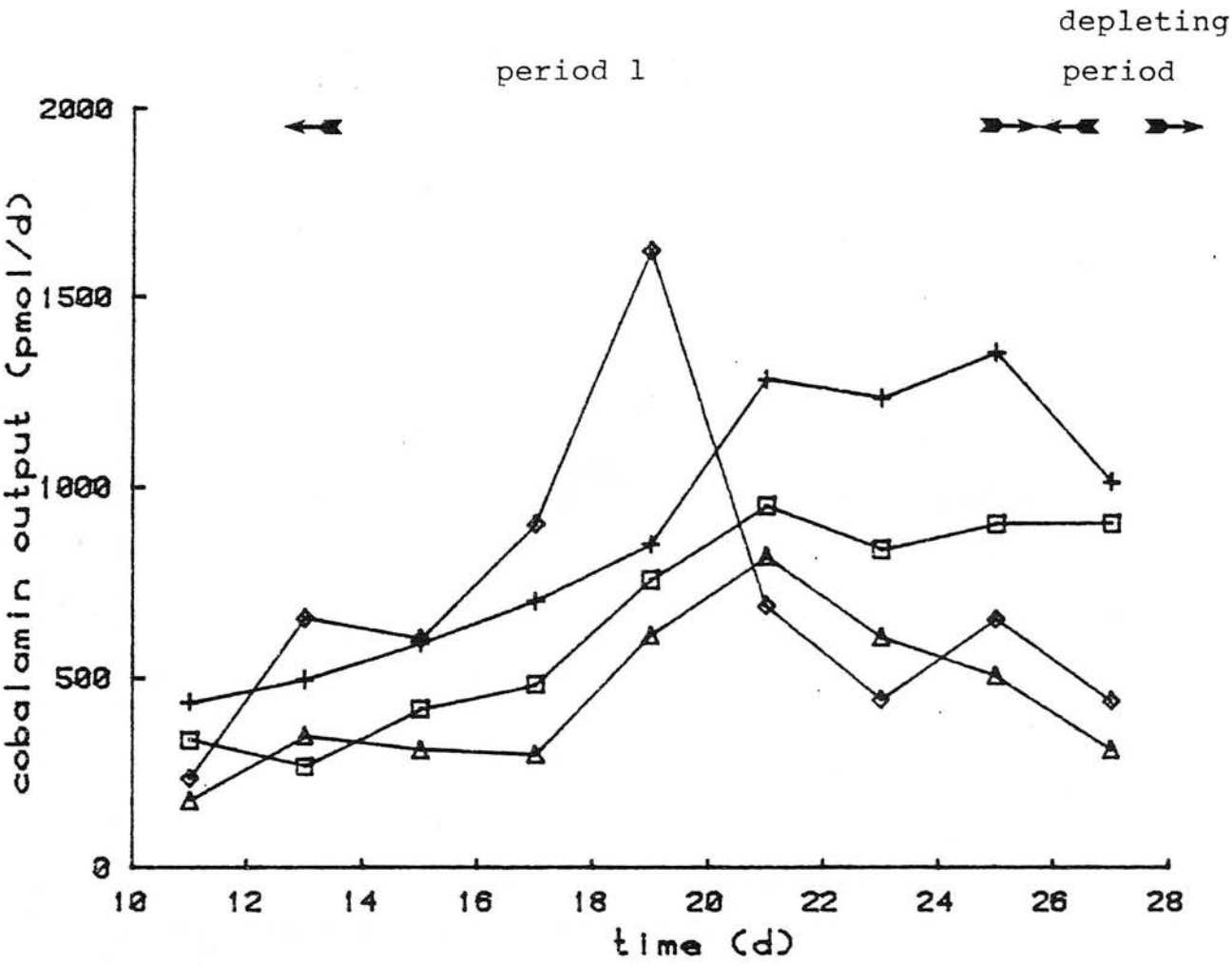


FIG. 4.6 Experiment 2b. Cobalamin outputs from vessels 1-4 given low cobalt hay(1) at 7 g DM/d and supplemented with cobalt during d 13-25 (period 1). (supplements: vessels 1 (+) and 2 (△) = 47.5 nmol Co/d, vessels 3 (□) and 4 (◇) = 23.8 nmol Co/d)
Supplement removed during d 26-28.

TABLE 4.5 Experiments 2a and 2b. Cobalamin and analogue outputs (pmol/d) and the analogue: cobalamin ratio when low cobalt hay(1) given at 7 g DM/d to Rusitec vessels 1-4, unsupplemented (d 7, 9 and 11) and cobalt supplemented (period 1, d 21, 23 and 25).
(Cobalt supplements: vessels 1 and 2= 47.5 nmol Co/d, vessels 3 and 4= 23.8 nmol Co/d)

	Vessels			
	1	2	3	4
Cobalamin				
Unsupplemented	374	353	361	545
Supplemented	1290	643	897	594
Analogues				
Unsupplemented	4820	1330	1800	-
Supplemented	8170	9970	10200	7940
Analogue: cobalamin				
Unsupplemented	12.9	3.77	4.99	-
Supplemented	7.33	16.5	12.4	14.4

late cut) and Astra clover were fed to vessels 2, 3 and 4 respectively, at 7 g DM/d (Table 3.1, opposite p 98). The 3 new hays had Co concentrations similar to the minimum requirement for ruminants (ARC, 1980) of 1.86 µmol/kg DM (0.11 mg/kg DM) and were supplied by the Welsh Plant Breeding Station, Aberyswyth.

Throughout periods 1 and 2, samples of vessel fluid for analysis of Cbl and VFA content were procured via the 3-way tap. Analyses for total vitamin B12 (L.leichmannii assay, p 91) were undertaken on a limited number of these samples and the fermentations were monitored by regular determination of ADMD and AOMD. In addition, diurnal variation of the Cbl concentration in compartment 1 was studied in both periods by sampling frequently over 24 h on 2 separate days (d 20 and d 34).

Results

Effects of cobalt supplementation upon fermentation (period 1)

Co supplementation increased the Cbl output from the cultures steadily for about 9 d (Fig. 4.6). The equilibrium values for d 21-25 showed that supplementation with Co increased Cbl output in all 4 vessels (Table 4.5). Although the mean Cbl responses were proportional to Co supplementation, variation between vessels (Fig. 4.6) meant that differences between treatments were not significant (Table 4.5). However, when data from Experiment 2a were included, the relationship between Cbl outputs (y; pmol Cbl/d) and Co input (x; nmol/d) was

TABLE 4.6 Experiments 2a and 2b. Mean ± s.d. of VFA molar proportions (%), output (mmol/d), and the acetate: propionate ratio, for Rusitec cultures 1-4 given low cobalt hay(1) at 7 g DM/d, either unsupplemented or supplemented with cobalt at one of two levels.

	Unsupplemented (n=4)	Supplemented (n=2)	
		23.8 nmol Co/d	47.5 nmol Co/d
VFA molar proportions			
Acetic acid	64.4 ± 0.881	66.8 ± 0.141	65.7 ± 1.20
Propionic acid	19.9 ± 1.09	20.8 ± 0.354	19.9 ± 0.424
Total butyric acid	10.4 ± 1.04	8.20 ± 0.311	9.16 ± 1.19
Total valeric acid	5.34 ± 0.764	4.41 ± 0.262	5.39 ± 0.233
Acetate: propionate	3.25 ± 0.154	3.22 ± 0.0566	3.30 ± 0.0141
VFA output			
Total VFA	35.1 ± 2.40	36.0 ± 1.20	35.7 ± 1.84
Acetic acid	22.6 ± 1.48	24.0 ± 0.849	23.5 ± 1.63
Propionic acid	6.96 ± 0.603	7.46 ± 0.134	7.12 ± 0.53
Total butyric acid	3.63 ± 0.468	2.95 ± 0.212	3.27 ± 0.262
Total valeric acid	1.88 ± 0.321	1.59 ± 0.149	1.92 ± 0.0141

described by the regression equation:

$$y=12.9x + 370 \quad r=0.776 \quad d.f.=6$$

The efficiency of incorporating Co into Cbl was therefore 1.29 %. Similarly, analogue output (y; pmol/d) also increased when additional Co was given (Table 4.5) and was related to Co input (x; nmol/d) by the equation:

$$y=157x + 2780 \quad r=0.832 \quad d.f.=5$$

The conversion efficiency for analogues was therefore 15.7 %.

The implication of these 2 linear equations for Cbl output was that the proportion of Cbl in total vitamin B12 did not increase with increased Co input.

The effects of Co supplementation upon VFA synthesis and digestibility were analysed using the paired t-test. No significant differences were found in any of the VFA outputs, either between the 2 levels of supplementation or between supplemented and unsupplemented vessels (Table 4.6). However, at the highest level of Co supplementation there was a significant ($p<0.05$) decrease in the proportion of total butyric acid and a significant increase ($p<0.05$) in the proportion of acetic acid, when compared to the unsupplemented value (Table 4.6). Digestibility was unaffected by Co supplementation (App. 2.2).

During the 3 d transition between Co supplementation and the introduction of the different hays (d 26-28), Cbl output dropped in vessels 1, 2 and 4 and remained constant in vessel 3 (Fig. 4.6).

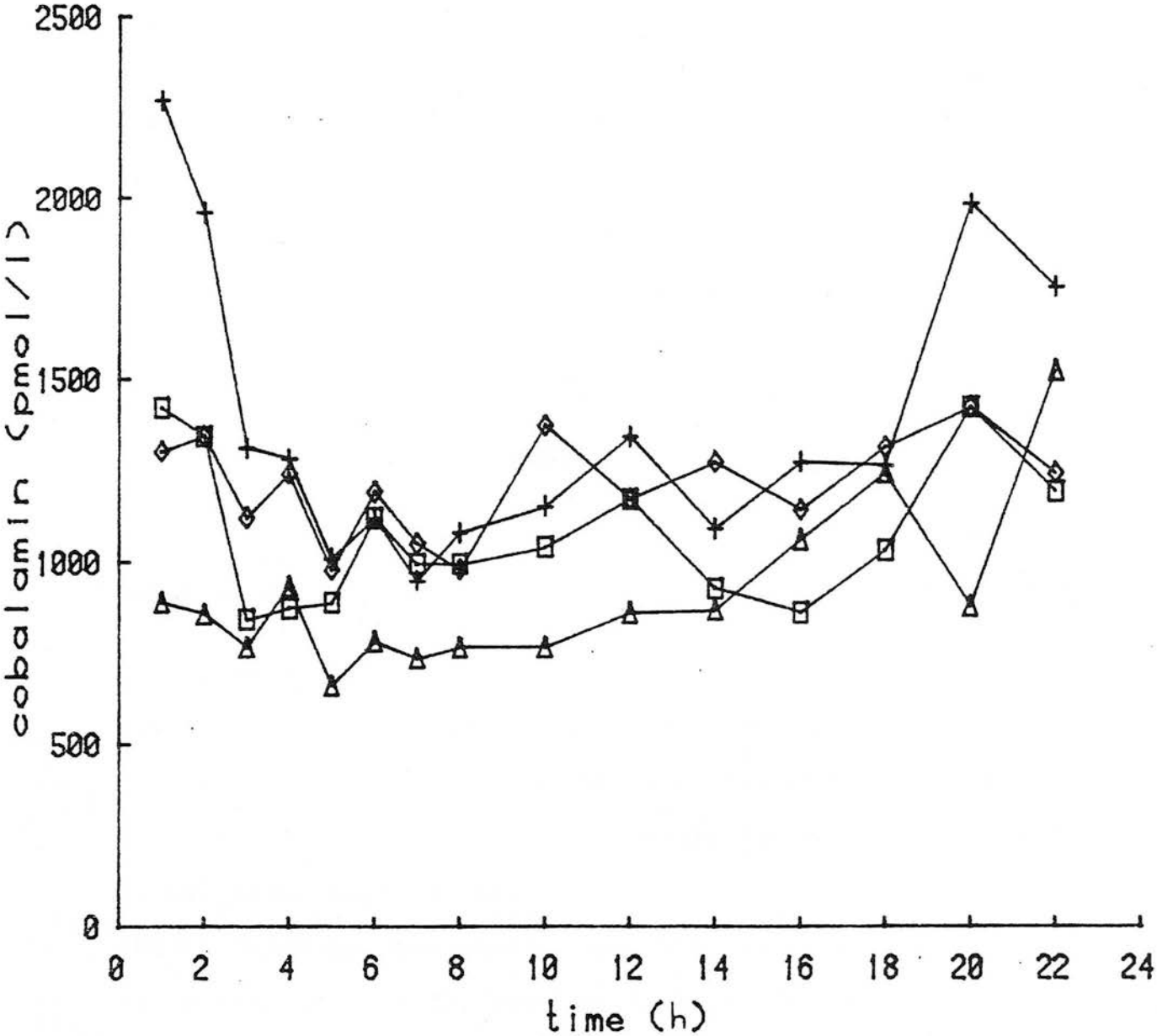


FIG. 4.7 Experiment 2b. Diurnal variation in cobalamin levels in compartment 1 of vessels 1-4, given low cobalt hay(1) at 7 g DM/d plus the following cobalt supplements on d 20 (period 1): vessels 1 (+) and 2 (△) = 47.5 nmol Co/d, vessels 3 (□) and 4 (◇) = 23.8 nmol Co/d.

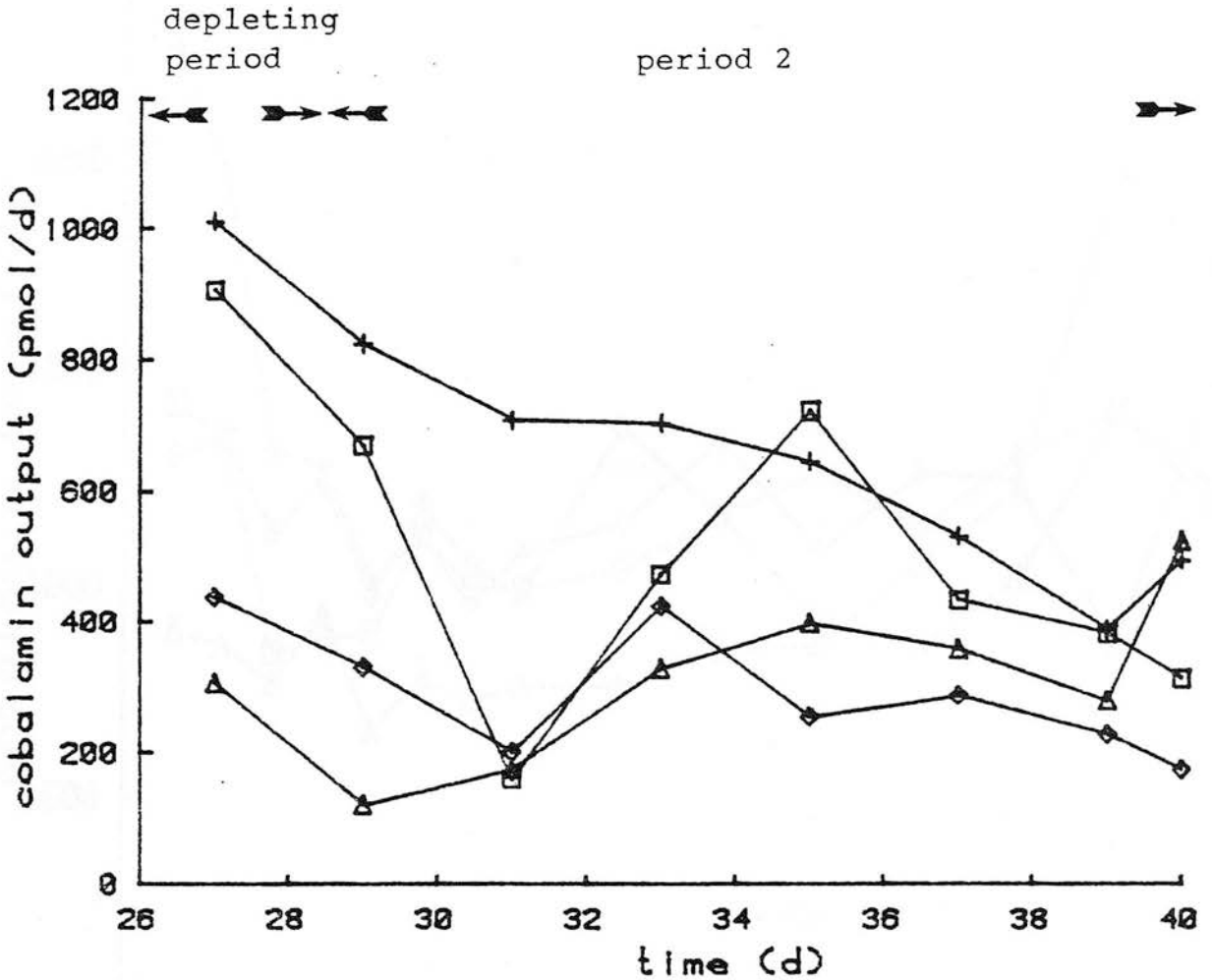


FIG. 4.8 Experiment 2b. Cobalamin outputs from vessels 1-4 when four different hays were given at 7 g DM/d during d 29-40 (period 2). (vessels: 1 (+) = low cobalt hay(1), 2 (△) = early ryegrass hay, 3 (□) = late ryegrass hay, 4 (◇) = clover hay)

TABLE 4.7 Experiment 2b. Cobalt content of four hays (nmol/kg DM), and mean output values (pmol/d) for cobalamin and analogues and the analogue: cobalamin ratio when the hays were given to Rusitec cultures at 7 g DM/d on d 37, 39 and 40. Efficiencies for the incorporation of cobalt (%) into cobalamin or analogues are in parentheses.

	Substrate			
	Mixed, low cobalt hay	Augusta ryegrass (early)	Augusta ryegrass (late)	Astra clover
Cobalt	543	1900	2300	1670
Cobalamin	471 (12.4)	386 (2.90)	376 (2.34)	229 (1.96)
Analogue	4460 (117)	4060 (30.5)	3560 (22.1)	2650 (22.6)
Analogue: cobalamin	9.47	10.5	9.47	11.6

Diurnal variation of cobalamin levels in compartment 1 (period 1) There were noticable differences between the vessels in Cbl levels over the period 1-5 h (Fig. 4.7); at 1 h, vessel 1 had the highest value and showed the fastest rate of decline. For the period 5-22 h, Cbl concentration increased significantly with time for vessels 1 ($p<0.01$), 2 ($p<0.01$) and 4 ($p<0.05$), as determined by the linear regression coefficients; the increases in Cbl concentration being 44.3, 36.2 and 16.4 pmol Cbl/l/h respectively. Different levels of Co supplementation did not produce different diurnal patterns. As in Experiment 2a, estimates of the Cbl contents of the reintroduced washings were made; the values being 1520, 439, 625 and 455 pmol Cbl for vessels 1-4 respectively. These correlated ($p<0.05$) with the mean Cbl output values in Table 4.5. Determination by the area method gave estimates for daily production of 1420, 967, 1090 and 1230 pmol for vessels 1-4 respectively.

Effects of different hays upon fermentation (period 2) Cbl outputs decreased initially from the high levels achieved during supplementation, but were considered to have equilibrated by d 37 (Fig. 4.8), as were analogue outputs (App. 2.1). The mean values for Cbl and analogue outputs for the period d 37-40 (n=3) are given in Table 4.7; they did not reflect the different levels of Co in the hays, maximal output coming from the hay(1) with the least Co, but the ranking of outputs from the different hays was the same for both Cbl and analogues.

Outputs of both Cbl and analogue /g OM digested correlated negatively with the ash content of the 4 hays. For Cbl, the relationship of output (y; pmol/g OM digested) to ash

TABLE 4.8 Experiment 2b. Mean VFA parameters and ADMD for different hays given during period 2 at 7 g DM/d to Rusitec cultures on d 37, 39 and 40. (Substrate: vessel 1= mixed low cobalt hay(1), vessel 2= Augusta ryegrass (early), vessel 3= Augusta ryegrass (late), vessel 4= Astra clover)

	Vessel			
	1	2	3	4
VFA molar proportions (%)				
Acetic acid	66.5	65.5	69.6	68.1
Propionic acid	20.5	20.7	19.3	18.0
Total butyric acid	9.79	10.5	8.36	9.59
Total valeric acid	4.23	3.61	2.60	4.38
Acetate: propionate	3.24	3.16	3.61	3.78
VFA output (mmol/d)				
Total VFA	38.8	41.5	42.7	31.3
Acetic acid	25.8	27.2	29.7	21.3
Propionic acid	7.95	8.59	8.26	5.62
Total butyric acid	3.80	4.34	3.57	3.00
Total valeric acid	1.64	1.50	1.11	1.37
ADMD	0.644	0.762	0.751	0.767

content (x; g/kg), when data from Experiment 2a were included, was:

$$y=-3.78x + 367 \quad r=0.790 \quad d.f.=6$$

~~and for analogues the relationship was:~~

~~$y=-89.8x + 7,870 \quad r=0.765 \quad d.f.=6$~~

Conversion efficiencies ranged from 1.96-12.4 % for Cbl synthesis and from 22.1-117 % for analogue synthesis. The efficiencies of conversion of Cbl from the ryegrass and clover hays, derived from output values (Table 4.7), were similar to those from inorganic Co and lower than the conversion value from the low Co hay(1), which gave an efficiency of 12.4 %. Levels of efficiency for the synthesis of analogues were noticeably lower for the ryegrass and clover hays than for the low Co hay(1) (Table 4.7) and were similar to the efficiencies during Co supplementation in period 1.

VFA production from the 4 different hays in period 2 was characteristic of a roughage fermentation (Table 4.8). Fermentation of the 2 ryegrass hays produced the highest outputs of total VFA, acetic and propionic acids; while for the clover hay, all VFA outputs were lower when compared to the low Co hay(1) (Table 4.8). Values for ADMD and AOMD (App. 2.2) were higher for the ryegrass and clover hays than for the low Co hay(1) and were approximately 6 % higher than the predicted <u>in vitro</u> values (Table 3.1).

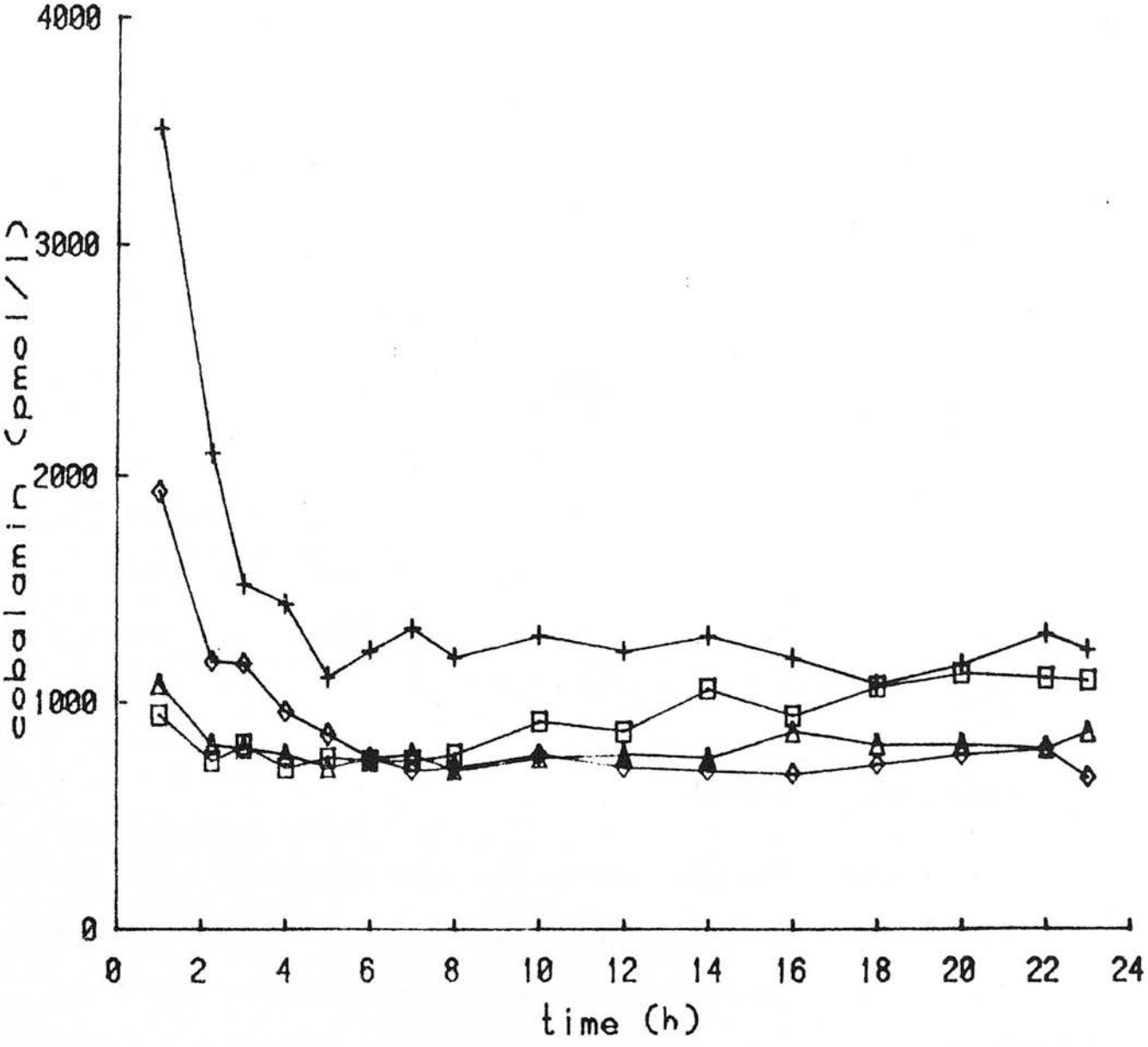


FIG. 4.9 Experiment 2b. Diurnal variation in cobalamin levels in compartment 1 of vessels 1-4, given four different hays at 7 g DM/d on d 34 (period 2).
(vessels: 1 (+) = low cobalt hay(1), 2 (△) = early ryegrass hay, 3 (□) = late ryegrass hay, 4 (◇) = clover hay)

Diurnal variation of cobalamin levels in compartment 1 (period 2)

The diurnal variations in Cbl concentration for the 4 different hays on d 34 are detailed in Fig. 4.9. The high early Cbl concentration in the vessel given the low Co hay(1) was associated with a high output during this period 2 (App. 2.1), but the different levels for the 4 hays did not reflect their Co content. Levels in all the vessels decreased from the first hour and stabilised after 5 h. Levels of Cbl from 5-23 h were much less variable for all 4 hays, than the values obtained when hay(1) was supplemented with Co in period 1 (see Figs. 4.7 and 4.9). Determination of the Cbl contents of the washings gave values of 2350, 444, 413 and 1680 pmol Cbl for the hay(1), early and late ryegrasses and clover hays respectively. For vessels 1, 2 and 4 these were higher than the values obtained during Co supplementation (period 1). Calculation of the daily Cbl production on d 34 by the area method gave values of 1490, 843, 978 and 889 pmol for vessels 1-4 respectively. The washings of the fully digested feed (48 h), taken from vessels 3 and 4 on d 40, contained 1490 and 1290 pmol Cbl by direct analysis, in total volumes of 110 ml.

Discussion

Effects of cobalt supplementation upon fermentation (period 1)

Co supplementation increased Cbl synthesis in all vessels and analogue production was increased in the 3 vessels for which data were complete. Tressol and Lamand (1979) found that the total vitamin B12 concentration in the Co-deficient rumen responded to Co supplementation within 8 h and was maximal after 4 d. The response in Rusitec took longer and was not uniform (Fig. 4.6). Cbl and analogue outputs from vessels 1-3 achieved equilibrium after 9 d of supplementation. For vessel 4, the maximum Cbl output was reached after 7 d (Fig. 4.6) and subsequent values were considerably lower, while analogue outputs in this vessel appeared to have equilibrated after 21 d. However, no other irregularities in relation to this vessel were noted, e.g. in artificial saliva (hence Co) input, digestibilities or VFA production.

The difference in the time taken for equilibration during Co supplementation in this study and that of Tressol and Lamand (1979), who examined homogenised rumen contents, might reflect the differences between *in vitro* and *in vivo* experimentation. Physical differentiation between compartments in Rusitec would have delayed the increase in Co levels in compartment 2 during this period and a time lag may have been introduced through the retention of Cbl within compartment 2, i.e. the same phenomenon suggested on p 120, but in this instance operating during Co-repletion. This may have been amplified by the microbes absorbing greater levels of Cbl, due to depleted stores, after the period of low Co input.

The large variation in both Cbl and analogue output values precluded significant treatment differences. Both Gawthorne (1970a) and Hedrich *et al.* (1973) have found Cbl and analogue synthesis in the rumen to vary greatly for a given dietary Co level.

The conversion efficiency in this experiment, of 1.29 % for Cbl synthesis from the Co supplement, derived from pooled data from Experiments 2a and 2b, agrees with that obtained in Experiment 1 (1.95 %). Values for the conversion efficiencies of Co into Cbl, from *in vivo* work using a roughage diet supplemented with inorganic Co, have been slightly greater (3.05-10.7 %, Table 1.2). The efficiency of incorporation of inorganic Co into Cbl was found, in Rusitec, to be lower than that for the organic Co supplied in the hay; whereas Hedrich *et al.* (1973) found the reverse to occur *in vivo* at an equivalent Co input. The efficiency of incorporation of Co from the Co supplement into analogues was lower (15.7 %) than that from the Co-deficient hay (69.8 % in Experiment 2a), a trend noted by Hedrich *et al.* (1973) who reported values of 77.2 % and 41.8 % for Co-deficient and Co-supplemented roughage diets respectively.

The proportion of Cbl to analogue (1:12.2) synthesised from the Co supplement did not change with the increased Co input and this was similar to that from the hays (1:9.47-11.6; Table 4.7); this is not the generally expected response (Hine and Dawbarn, 1954; Gawthorne, 1970a; Hedrich *et al.*, 1973).

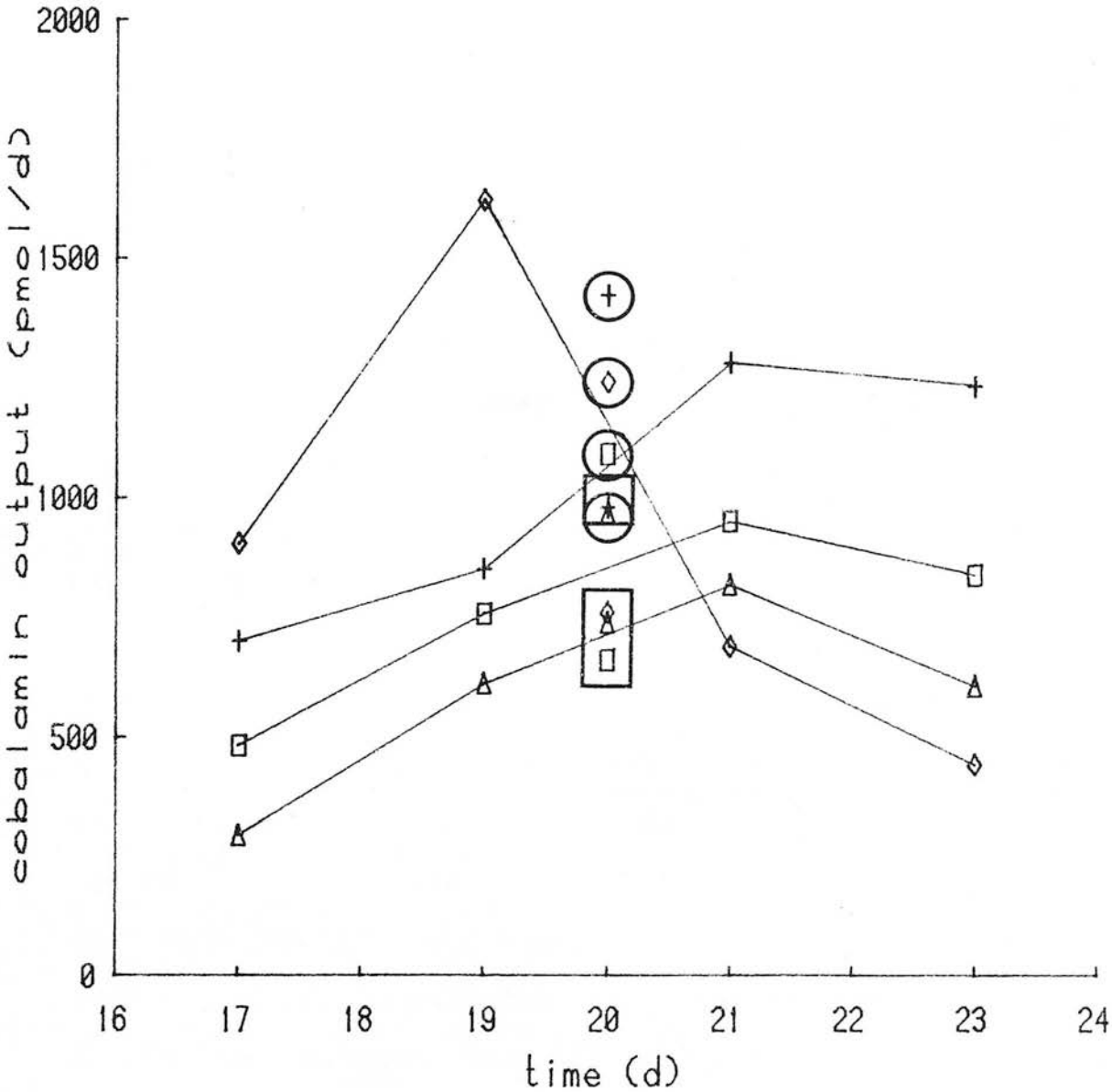


FIG. 4.10 Experiment 2b. Cobalamin output values from vessels 1-4 on d 17, 19, 21 and 23, compared with productivity values on d 20 calculated from the diurnal samples by;

a) the area method (encased in circle),

b) extrapolation of the stable diurnal values to 24 h and determination of an output value (encased in square).

The importance of the altered proportions of acetic and total butyric acids during Co supplementation requires confirmation.

Withdrawal of the Co supplement decreased Cbl output by 21.7, 52.3 and 26.4 % within 2 d for vessels 1, 2 and 4 respectively. Assuming a single compartment system of 800 ml capacity, a dilution rate of 0.75 /d and complete, instantaneous mixing then the Co concentration should have been reduced by 90 % within 2 d. However, the physical and microbial differences between compartments plus sequestration and recycling of Cbl in compartment 2 probably slowed the rate of decrease in Cbl output.

Diurnal variation of cobalamin levels in compartment 1 (period 1) On d 20, during the period 1-22 h, 2 phases were evident (Fig. 4.7). Cbl levels decreased up to 5 h and estimations of the Cbl added in the reintroduced washings (1520, 439, 625 and 455 pmol for vessels 1-4 respectively) again confirmed the influence of compartment 2. The initial decline in Cbl values was again considered to be due to the sequestration of this added Cbl by the feed matrix. The estimates of Cbl in the washings did not correlate with estimates of Cbl production, as determined by the area method, possibly because the initial peaks were poorly defined in 3 vessels (Fig. 4.7). "Area outputs" were again greater than outputs derived from Cbl levels at the end of the diurnal sampling, which agreed with the results for the adjacent daily samplings (Fig. 4.10).

Cbl levels increased with time over the period 5-22 h for vessels 1, 2 and 4 (Fig. 4.7), whereas in Experiment 2a they remained constant. The increasing levels indicated that the synthesis of Cbl in compartment 1 plus the flow of Cbl from compartment 2 was greater than the effect of dilution by the artificial saliva plus any flow of Cbl into compartment 2. For vessels 1 and 2 the increases during 5-22 h reflected the steadily increasing outputs over the period 17-21 d (Fig. 4.10).

Estimated Cbl production on d 20 of 1420, 967, 1090 and 1230 pmol for vessels 1-4 respectively, compared to mean daily outputs from d 21, 23 and 25 of 1290, 643, 897 and 594 pmol respectively. This implies that the outputs based on vessel fluid analysis were underestimating daily production by between 9 and 51 %.

Supplementing compartment 1 with Co (period 1) contrasts with the normal provision of Co in the feed, which is then released into compartments 2 and 3. When an unsupplemented regime is employed, Cbl will flow into compartment 1 either free or bound to microbial matter, but the latter flow is likely to be small because microbial movement away from the nutrient source will be minimal (Czerkawski, 1979). Consequently, both Co and Cbl would be expected to be partially "trapped" within compartments 2 and 3, but Cbl levels in compartment 1 would eventually equilibrate. Provision of Co directly into compartment 1 during supplementation may have caused some microbes to migrate towards the Co and this may have caused the wider diurnal variation (Fig. 4.7 vs. 4.4). Furthermore, a reduction in

"available" Co to the microbial populations of compartments 2 and 3, may explain the reduced efficiencies of Cbl synthesis when Co was infused. Conversion efficiencies might be greater for an equivalent Co input in a more homogenous system, such as the rumen.

Effects of different hays upon fermentation (period 2)

Output values for both Cbl and analogues had equilibrated 9 d after the introduction of the different substrates (d 37). This is substantiated by the output values for vessel 1, which were similar to the those in Experiment 2a, when the same low Co hay was used (App. 2.1).

The 3 hays employed in vessels 2-4, while having Co contents up to 4 times that of the low Co hay(1), all produced lower mean Cbl and analogue outputs. The negative relationship of both Cbl and analogue output with ash content suggested that other elements may have inhibited the microbial uptake of Co. It is noteworthy that manganese and magnesium are believed to share a common transport pathway with Co in E.coli (Nelson and Kennedy, 1971) and Bacillus subtilis (Scribner et al., 1974) and both these elements affect microbial Co utilisation (Pfander et al., 1966; Martinez, 1972).

Bigger et al. (1976) suggested that dietary precursors other than Co may be important in both Cbl and analogue synthesis, as certain bases increased the production of many corrins. However, good quality hays, such as those used in this study would be expected to have provided such precursors in similar amounts to those suppied by the low Co hay(1) (Table 3.1), yet the Cbl outputs were less.

For analogue outputs, the hay used in vessel 1 had a conversion efficiency in excess of 100 %, which suggested that the previous period, in which Co supplements were used, may have influenced the results or that contamination was occurring in vessel 1 only. If carryover was involved then it might be expected to have occurred in all 4 vessels, as analogue outputs were similar during Co supplementation (period 1). However, the washings from vessel 1 contained 3 times more Cbl than those from the other vessels. Furthermore, the change of hay in vessels 2-4 may have altered the composition and numbers of microflora and the lower ADMD of the low Co hay(1) might have been an important factor in this. Because of its lower digestibility the mass of undigested low Co hay(1) left after 48 h would have been 50 % greater than that of the other hays and so might have been capable of sequestering a significantly larger microbial population, giving a greater capacity for vitamin B12 synthesis and potential carryover effect. Although the volume of the washings (compartment 2) from the 48 h-digested feed on d 40 was 30 ml for all 4 hays there may have been differences in microbial composition, including Cbl content, and numbers.

The poor efficiency of Cbl synthesis from the Co in the highly digestible hays may have resulted from the rapid dispersal of the inherent Co giving a similar situation to that obtained by infusing the Co into compartment 1. Results for the efficiencies of analogue synthesis from the ryegrass and clover hays in Rusitec, covered a similar range as reported values for Co-adequate rations (Tables 1.2 and 1.3).

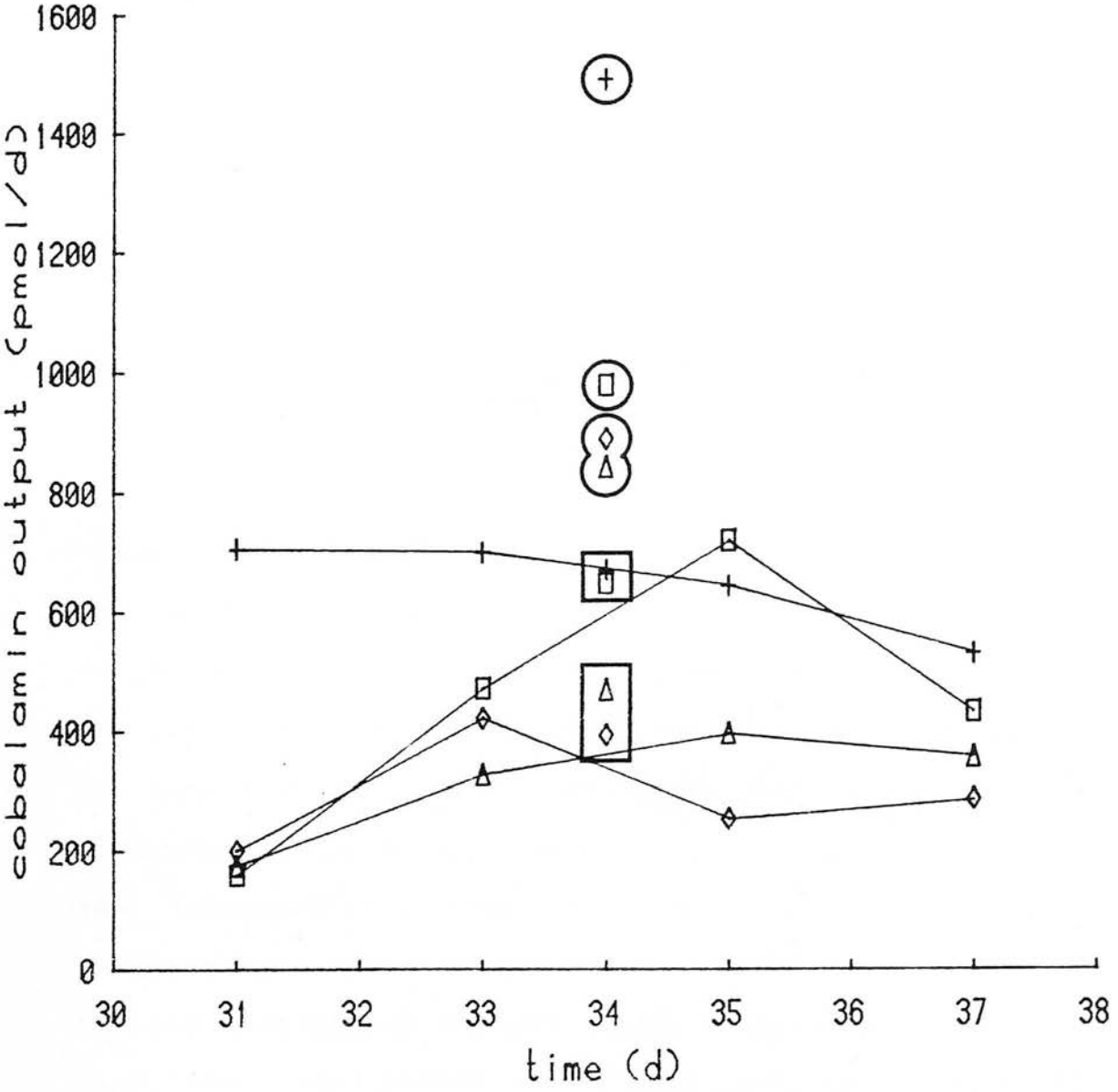


FIG. 4.11 Experiment 2b. Cobalamin output values from vessels 1-4 on d 31, 33, 35 and 37, compared with productivity values on d 34 calculated from the diurnal samples by;

a) the area method (encased in circle),

b) extrapolation of the stable diurnal values to 24 h and determination of an output value (encased in square).

At the start of d 30, 2 fresh feed bags (early and late ryegrass hays) were put into vessel 2 by accident, alongside the 24 h-digested feed, while vessel 3 was occupied by a single bag of 24 h-digested feed only, i.e. it was "starved". This mistake was rectified 24 h later, but it probably accounts for the low Cbl (Fig. 4.8) and VFA outputs from vessel 3 on d 31.

Values for AOMD of the ryegrass and clover (App. 2.2) were greater than the in vitro predicted values (Table 3.1). This was considered to be due to the sustained digestion (48 h) in Rusitec.

Diurnal variation of cobalamin levels in compartment 1 (period 2) The influence of Cbl, in the reintroduced washings, upon the diurnal levels was again shown to be considerable for some vessels and the calculated Cbl content of these washings varied by a factor of 5, being maximal for the low Co hay(1). Direct assay of the washings from vessels 3 and 4 on d 40 confirmed that large quantities of Cbl were present. The differences between measurements of the Cbl output and Cbl production were greater than that found during Co supplementation (Figs. 4.10 and 4.11), possibly because of a shift in Cbl distribution from compartment 1 to compartment 2. Assuming that the area method is a good assessment of Cbl production, then resting output values are a considerable underestimate of productivity. The comparisons between "area" productivity and output allowed for corrections to be made for underestimating (Fig. 4.11; Ch. 8, p 208). From 5-23 h Cbl levels remained constant (Fig. 4.9) and this contrasted with period 1 (Fig. 4.7), when Co was infused.

Conclusions

1. The efficiencies of Cbl synthesis were lower for the Co infused into compartment 1 than for the endogenous Co of a low Co hay (1.29 % vs. 11.1 ± 2.20 %) and also lower for 3 hays of greater digestibility and Co content than the low Co hay(1) (2.40 ± 0.473 % vs. 11.1 ± 2.20 %). These differences may be due to the displacement of Co from compartment 2, either by infusing the element into compartment 1 or by the rapid digestion of the matrix. For the different hays, Cbl and analogue outputs were not determined solely by Co content.
2. Sampling Rusitec from within the vessel after 24 h was again found to underestimate daily production and the advantages of using the effluent as a measure of productivity became apparent.
3. Variability of output for vitamin B12 occurred both between vessels and between days at any given Co input; but this did not represent instability in the culture, as measured by VFA synthesis and digestibility.

CHAPTER 5

EXPERIMENT 3. THE EFFICIENCY OF VITAMIN B12 SYNTHESIS IN RUSITEC, WHEN BARLEY IS USED AS THE FOOD SUBSTRATE

Introduction

The proportion of Cbl in total vitamin B12 synthesised by rumen microbes has been found to decrease with an increased percentage of concentrate in the diet (Sutton and Elliot, 1972). It was therefore decided to use barley as the food substrate in Rusitec to produce a "propionate" fermentation typical of a concentrate diet. Using barley of a low Co content (Table 3.1), the response of vitamin B12 synthesis to supplementation with inorganic Co was assessed. Propionate metabolism requires cobamide as a coenzyme in both mammals and microorganisms (Babior, 1975); consequently, low Cbl levels might influence propionate metabolism by this pathway. The effects of Co supplementation on fermentation patterns were therefore examined.

In aerobic bacteria, riboflavin has been found to be a precursor of the base 5,6 dimethylbenzimidazole (Renz et al., 1979), which is a characteristic component of Cbl. Supplementation of the barley, which is known to be low in riboflavin (McDonald et al., 1977), with riboflavin was therefore included as a variable in this experiment to ascertain whether Cbl synthesis could be increased in an anaerobic system.

TABLE 5.1 Experiment 3. Experimental design for vessels 1-4 to investigate the effect of cobalt and riboflavin supplements upon vitamin B12 synthesis in cultures given low cobalt barley as the substrate.

		Vessels			
		1	2	3	4
Period	Substrate				
Initial	hay(1)	<----------no supplementation---------->			
1	barley	<----------no supplementation---------->			
2	barley	+ 11.9 nmol Co/d		+ 23.8 nmol Co/d	
3	barley	+ 47.6 nmol Co/d		+ 95.2 nmol Co/d	
4	barley	no cobalt ***		+ 95.2 nmol Co/d	***

*** =2.66 μmol riboflavin/d.

Experimental procedure

Barley of a low Co content (185 nmol Co/kg DM, equivalent to 0.0109 mg/kg DM, Table 3.1) was obtained from East Nisbet Farm, Crailing, nr. Jedburgh. Several changes were made from the previous experimental procedure, when initiating the rumen cultures, to try to hasten the attainment of equilibrium in Rusitec.

The inoculum was pooled rumen contents taken from 4 fistulated Blackface sheep fed upon the low Co hay(1) used previously; this hay was also used initially in Rusitec to establish a fermentation. The experimental design is outlined in Table 5.1. After 6 d the food substrate was changed to crushed barley given at 7 g DM/d. The unsupplemented barley was fed for 17 d (period 1, d 7-23) to establish equilibrium and assess the uniformity of vitamin B12 and VFA production.

In period 2, vessels were paired for the determination of vitamin B12 production at 2 levels of Co, which was supplied in the artificial saliva; 11.9 nmol/d (equivalent to 0.10 mg Co/kg DM) was infused into vessels 1 and 2 and 23.8 nmol/d (0.20 mg Co/kg DM) into vessels 3 and 4, for 14 d (d 24-37). In period 3, Co supplementation levels were increased 4-fold to 47.6 nmol/d (0.40 mg Co/kg DM) and 95.2 nmol/d (0.80 mg Co/kg DM) for each pair of vessels, for a further 14 d (d 38-51).

In the final phase, period 4 (d 52-57), the effect of riboflavin supplementation upon Cbl synthesis was assessed at 2 levels of Co input. Supplementation with Co was

terminated in vessels 1 and 2, but continued at 95.2 nmol/d in vessels 3 and 4. After allowing 3 d for the Co levels in vessels 1 and 2 to fall, riboflavin was added to vessels 1 and 4 at feeding, at the level of 2.66 µmol/d for 3 d.

In view of the results from Experiment 2, productivity from the vessels was measured as effluent output. Effluent from each vessel was collected in flasks containing 5 ml 0.06 M sodium cyanide to inhibit further bacterial activity and to convert the different forms of vitamin B12 to the cyano forms (p 7). Throughout the experiment, samples were taken from the 24 h effluent collection for analysis of Cbl and VFA and, on certain days, for vitamin B12 analogues; the remaining effluent was discarded. Samples of "washings" from the digested food were taken and analysed for Cbl on d 51 and d 57. In addition, samples of vessel fluid were taken via the 3-way tap in periods 1 (d 14) and 3 (d 48) to ascertain diurnal variation in Cbl production before and during periods of Co supplementation. Samples were taken immediately after feeding, as the importance of post-feeding samples had been revealed in previous experiments. The dilution rate was maintained at 0.75 /d during the experiment.

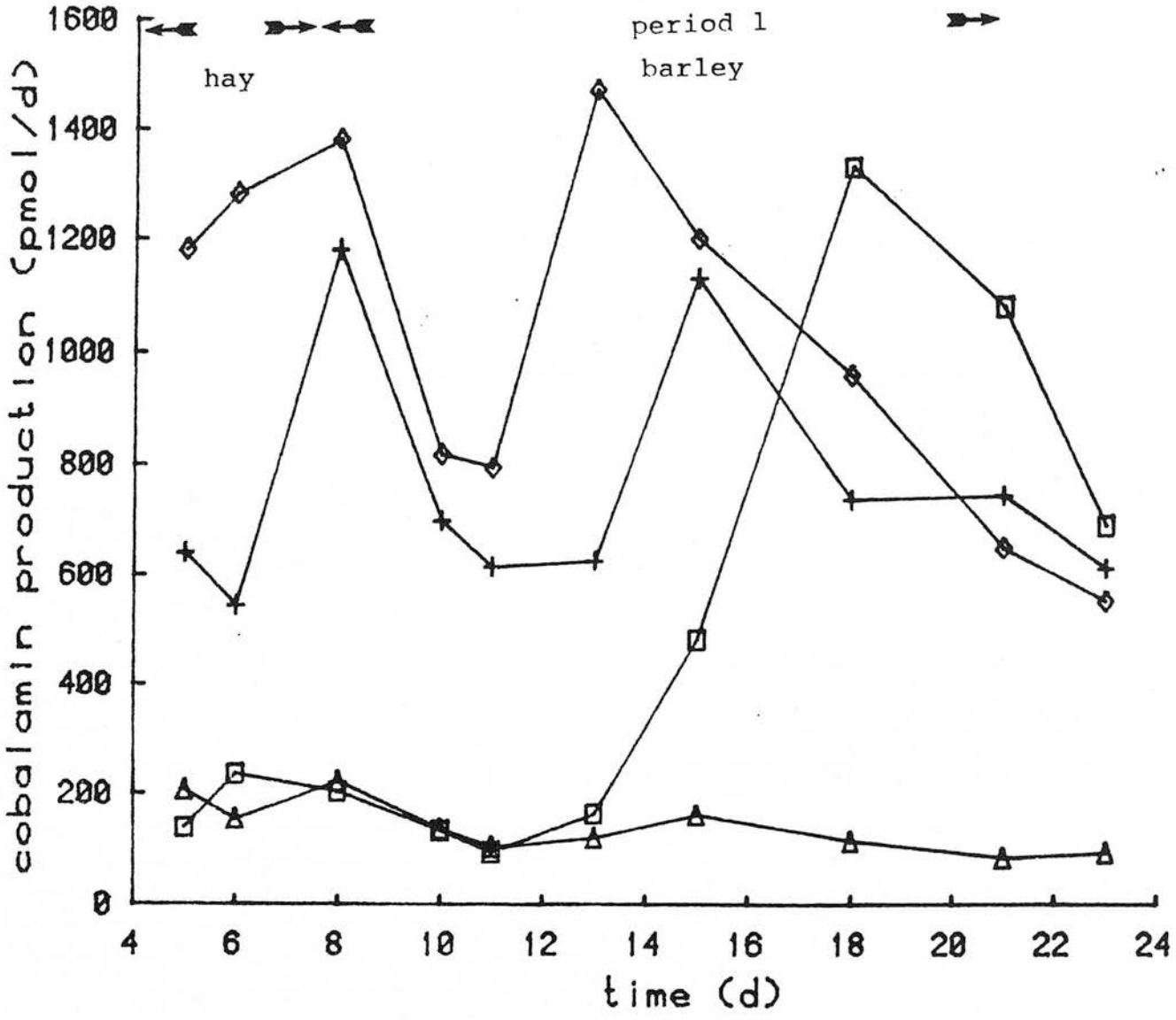


FIG. 5.1 Experiment 3 Cobalamin production values (pmol/d) from vessels 1-4 given 7 g DM low cobalt hay(1)/d up to d 6, then given 7 g DM barley/d during period 1 (d 7-23).
(vessels: 1 = + , 2 = △ , 3 = □ , 4 = ◇)

TABLE 5.2 Experiment 3. Mean cobalamin and analogue production (pmol/d) during the preliminary period (hay, 7 g DM/d) and period 1 (barley, 7 g DM/d). Mean values were derived from d 5, 6 in the preliminary period for both Cbl and analogues; while in period 1, d 18, 21 and 23 were used for Cbl and d 23 only for analogues.

	Vessel				
	1	2	3	4	mean ± s.d.
Diet					
Cobalamin					
hay	592	181	187	1230	548 ± 494
barley	699	96.1	1030	721	637 ± 391
Analogues					
hay	7850	4450	5910	5010	5810 ± 1490
barley	886	465	1570	728	912 ± 472

TABLE 5.3 Experiment 3. Conversion efficiencies (%) of cobalt into cobalamin and analogues during the preliminary period (hay, 7 g DM/d) and period 1 (barley, 7 g DM/d). The values are derived from mean production levels (given in TABLE 5.1).

	Vessel				
	1	2	3	4	mean ± s.d.
Diet					
Cobalamin					
hay	15.6	4.76	4.92	32.4	14.4 ± 13.0
barley	54.2	7.45	79.9	55.9	49.4 ± 30.3
Analogues					
hay	207	117	156	132	153 ± 39.4
barley	68.7	36.1	122	56.4	70.8 ± 36.7

Results

Preliminary period and resting values (period 1)

Vitamin B12 synthesis For both hay and barley diets, variation in Cbl (Fig. 5.1) and analogue production between vessels was large (Table 5.2), vessel 2 having a consistently low level of Cbl production and vessels 1 and 4 high levels of Cbl production. The production of Cbl and analogues during period 1 are summarised in Table 5.2. Inter-day variation for Cbl production was also large when barley was fed, with the exception of vessel 2. Cbl production increased markedly in vessel 3 after d 13, while production in vessel 4 declined (Fig. 5.1).

As in Experiment 2, equilibration of analogue production appeared to take longer (17 d) than that of Cbl production (ca. 12 d, App. 3.1), therefore, analogue production on d 23 was used as the resting value. Mean values for Cbl production were unaffected by the change of diet from hay to barley, but analogue production decreased in all vessels when barley was fed (Table 5.2).

The efficiencies of Co incorporation into Cbl and analogues are given in Table 5.3. Notable were the high (>100 %) values for the synthesis of analogues from the hay diet. Inter-vessel variation was reflected in the high c.v. of the conversion efficiencies for Cbl and analogue production; the values being 90.3 and 25.8 % respectively in the preliminary period and 61.3 and 51.8 % respectively in period 1. The higher conversion efficiencies for Cbl in period 1, compared with the values for the preliminary period (Table 5.3), were due to the lower Co content of the

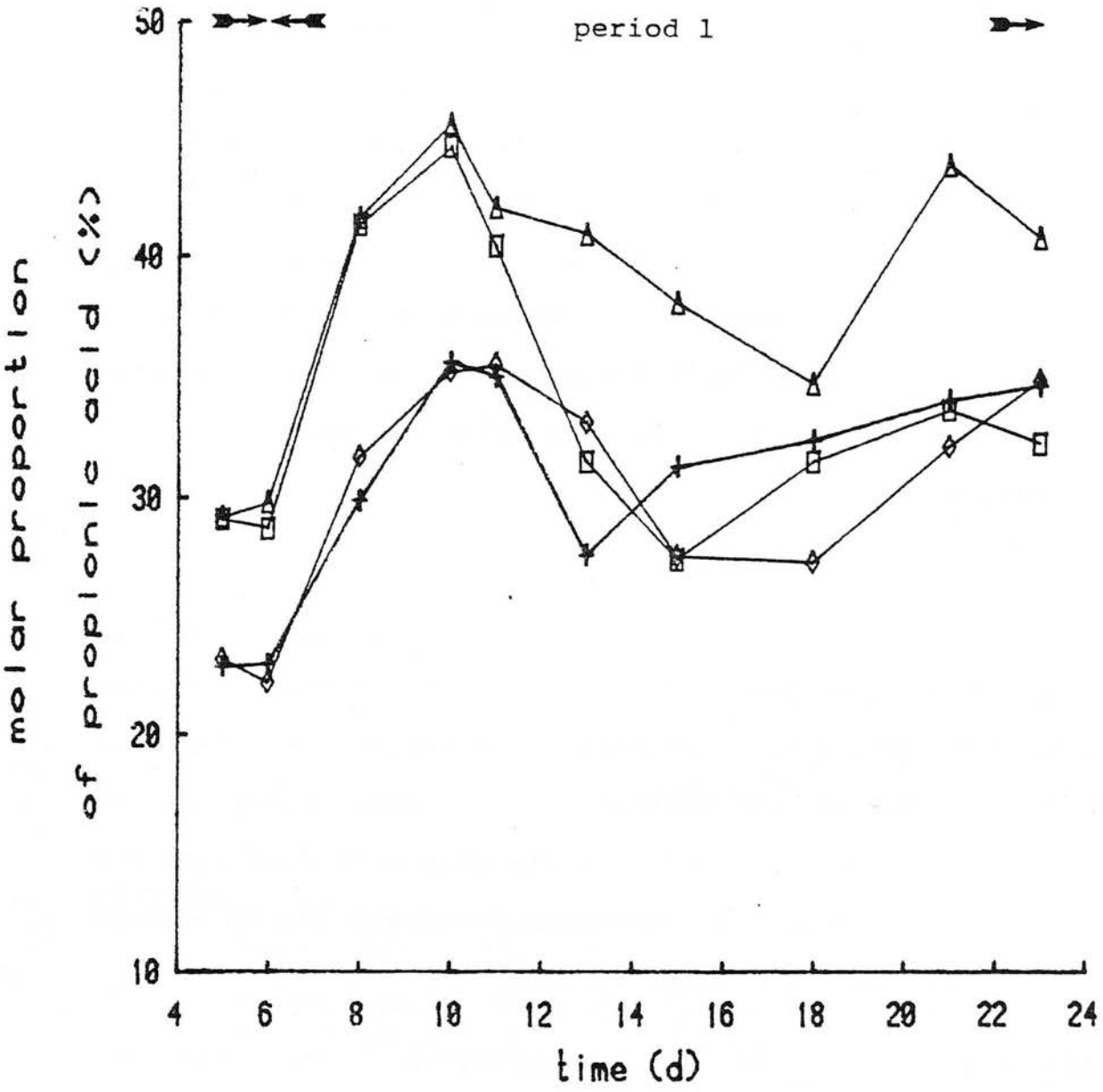


FIG. 5.2 Experiment 3. Molar proportions of propionic acid (%) in total VFA for effluent from vessels 1-4; given 7 g DM low cobalt hay(1)/d up to d 6, then 7 g DM barley/d during period 1 (d 7-23). (vessels: 1 = + , 2 = △ , 3 = □ , 4 = ◇)

TABLE 5.4 Experiment 3. Mean VFA molar proportions (%), acetate: propionate ratio and VFA production (mmol/d) in period 1 for vessels 1-4 given barley at 7 g DM/d. Mean values were derived from d 18, 21 and 28.

	Vessel				
	1	2	3	4	mean (1,3 and 4) ± s.d.
Molar proportions					
Acetic	30.7	26.6	33.5	31.5	31.9 ± 1.44
Propionic	33.6	40.5	26.7	32.6	31.0 ± 3.73
Total butyric	27.7	22.8	26.1	29.2	27.7 ± 1.55
Total valeric	7.87	10.2	8.04	7.86	7.92 ± 0.101
Acetate: propionate	0.914	0.657	1.26	0.966	1.05 ± 0.187
VFA production					
Total	43.7	50.1	38.2	40.7	40.9 ± 2.75
Acetic	13.4	13.3	12.8	12.8	13.0 ± 0.346
Propionic	14.7	20.3	12.4	12.8	13.3 ± 1.23
Total butyric	12.1	11.4	9.95	11.9	11.3 ± 1.19
Total valeric	3.44	5.10	3.07	3.20	3.24 ± 0.188

TABLE 5.5 Experiment 3. ADMD for low cobalt hay (7.22 g DM/d) given in the preliminary period and for the low cobalt barley (7.00 g DM/d) given in period 1, to vessels 1-4 of Rusitec.

	Vessel			
	1	2	3	4
Diet				
hay	0.627	0.574	0.617	0.625
barley	0.875	0.873	0.870	0.875

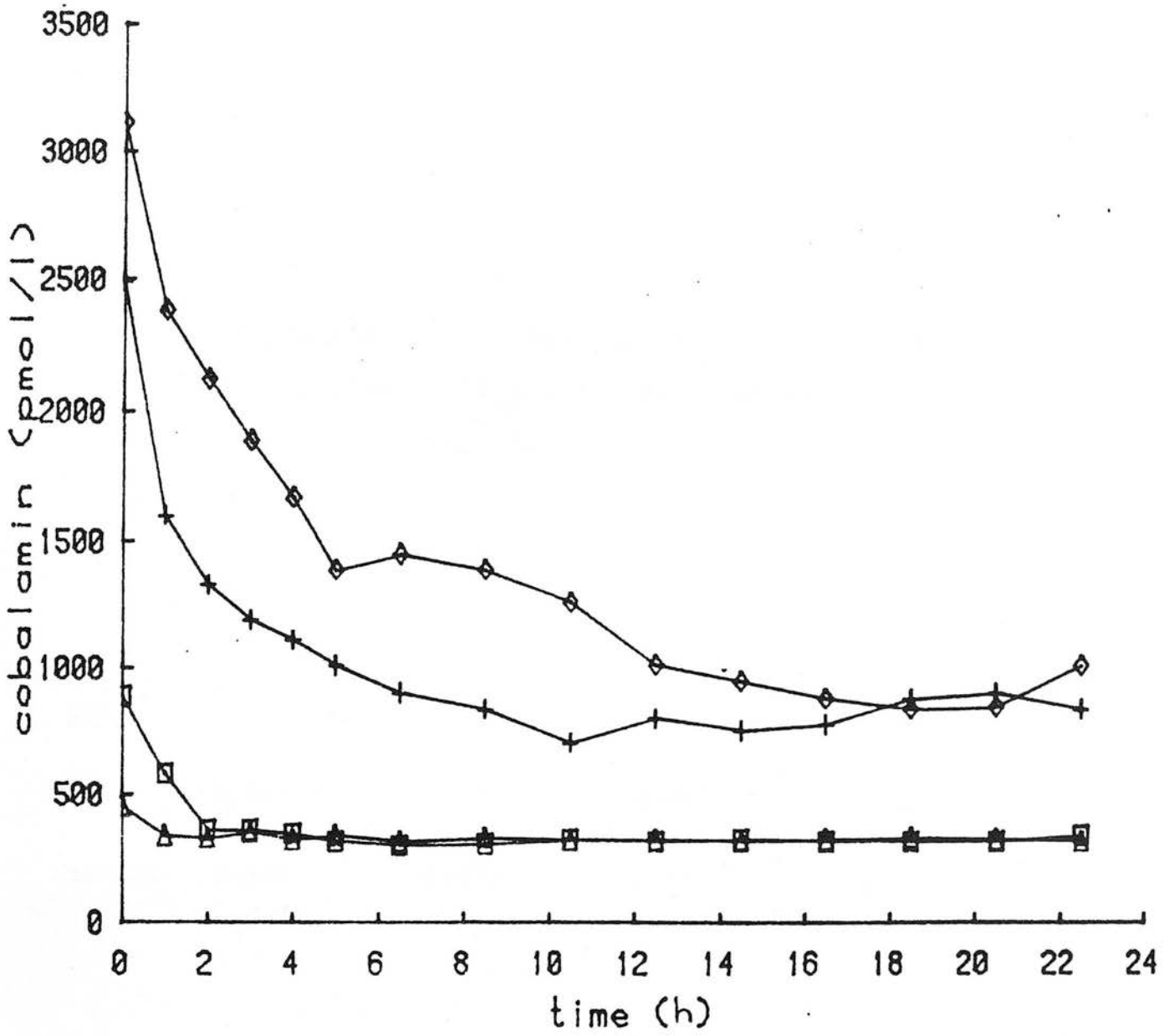


FIG. 5.3 Experiment 3. Diurnal variation in cobalamin levels in compartment 1 of vessels 1-4 given 7 g DM barley/d on d 14 (period 1).
(vessels: 1 = + , 2 = △ , 3 = □ , 4 = ◇)

barley (Table 3.1, opposite p 98); while the lower analogue production in period 1, compared with that in the preliminary period, accounted for the reduced conversion efficiencies (Table 5.3).

VFA synthesis and digestibility Introduction of the barley diet produced the expected change from an "acetate" to a "propionate" fermentation (Fig. 5.2), with the range of acetate: propionate ratios being reduced from 1.42-2.66 for the hay to 0.657-1.26 for the barley; but it did not immediately reduce the differences in propionate synthesis established between vessels when hay was fed. For the first 10 d the cultures in vessels 2 and 3 maintained fermentations producing a greater proportion of propionic acid than those in vessels 1 and 4 (Fig. 5.2), but for vessel 3 this proportion decreased between d 10 and d 14. Thereafter, the fermentation in vessel 2 continued to produce a higher proportion of propionic acid when compared to the other vessels and this difference was accompanied by a higher proportion of total valeric acid in the total VFA (Table 5.4). VFA proportions appeared to have achieved representative stable values by d 18, e.g. Fig. 5.2.

ADMD values were similar in vessels 1, 3 and 4 during period 1, with a slightly lower value for the substrate in vessel 2, but all were greater than those for the hay (Table 5.5).

Diurnal variation in cobalamin levels (period 1) The diurnal fluctuations in Cbl levels in vessels 1-4 on d 14 are shown in Fig. 5.3. Initially, vessels 2 and 3 had a

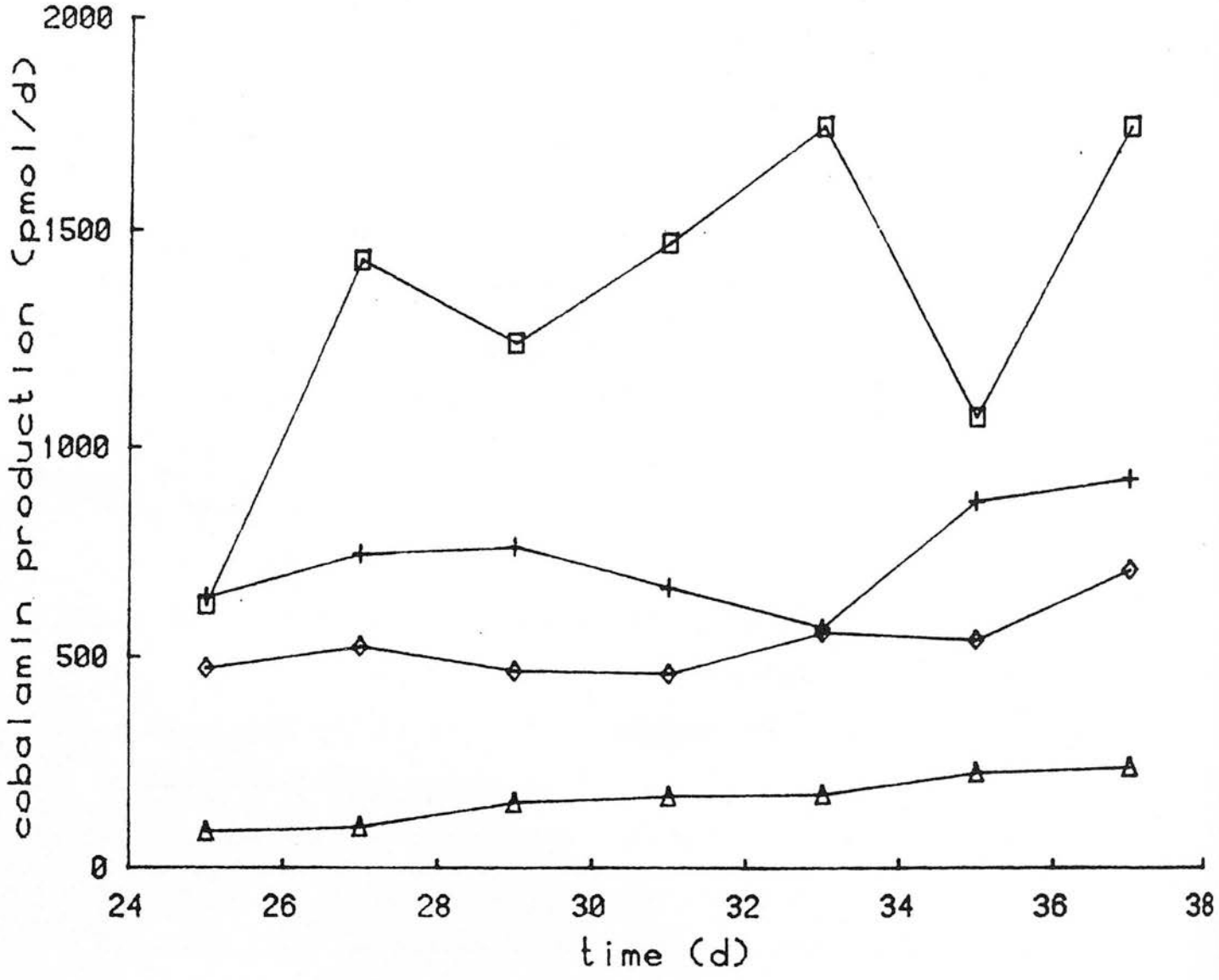


FIG. 5.4 Experiment 3. Cobalamin production values (pmol/d) from vessels 1-4 given 7 g DM barley/d and supplemented with cobalt (period 2, d 24-37). (supplements: vessels 1 (+) and 2 (△) = 23.8 nmol Co/d, vessels 3 (□) and 4 (◇) = 47.5 nmol Co/d)

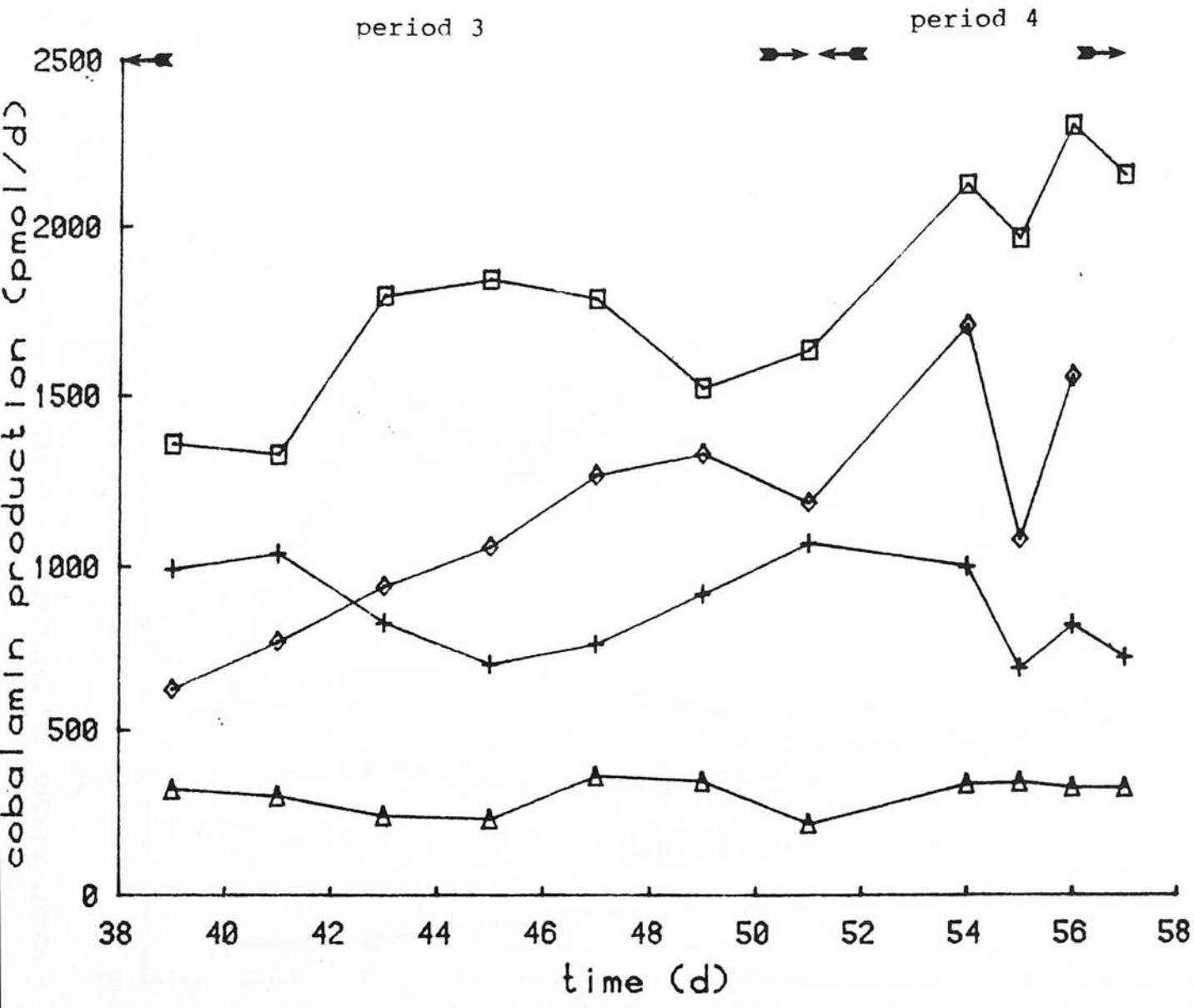


FIG. 5.5 Experiment 3. Cobalamin production values (pmol/d) from vessels 1-4 given 7 g DM barley/d and supplemented with cobalt (period 3, d 38-51) and/or 2.66 umol riboflavin/d (period 4, d 52-57). The supplements employed were:

	d 39-51	d 52-54	d 55-57
vessel 1 (+)	47.6 nmol Co/d	no cobalt	+ riboflavin
vessel 2 (△)	47.6 nmol Co/d	no cobalt	-----
vessel 3 (□)	95.2 nmol Co/d	95.2 nmol Co/d	95.2 nmol Co/d
vessel 4 (◇)	95.2 nmol Co/d	95.2 nmol Co/d	95.2 nmol Co/d + riboflavin.

TABLE 5.6 Experiment 3. Vessel responses in cobalamin and analogue production to four levels of cobalt supplementation of barley given at 7 g DM/d. Responses were the mean values for d 33, 35 and 37 for period 2 or d 47, 49 and 51 of period 3 less the resting values for period 1 given in TABLE 5.2.

Cobalt supplement (nmol/d)	Vessel response (pmol/d)			Mean response
Cobalamin				
11.9	vessel	1) 91	2) 116	104
23.8	"	3) 490	4)-119	186
47.6	"	1) 219	2) 211	215
95.2	"	3) 610	4) 539	575
Analogues				
11.9	vessel	1) 724	2)1480	1100
23.8	"	3)1940	4) 882	1410
47.6	"	1)2890	2)1810	2350
95.2	"	3)4080	4)4340	4210

low Cbl level compared with vessels 1 and 4, for which values were 4-fold greater. The time taken for the vessels to equilibrate reflected the initial Cbl levels, being 2 h for vessels 2 and 3 and 12.5 h for vessels 1 and 4 and the equilibrium values in vessels 1 and 4 were approximately twice those of the other vessels. The influence of the reintroduced washings upon the Cbl levels in compartment 1 immediately after feeding was recognised in Experiment 2; therefore, in order to accurately quantify this contribution, sampling from within the vessel was undertaken from 0 h onwards. Using the same procedure described in Experiment 2 (p 118), estimates of the Cbl contents of the reintroduced washings were 1280, 114, 398 and 1490 pmol for vessels 1-4 respectively. Estimates of the daily Cbl production, determined by the area method, were 950, 327, 344 and 1280 pmol/d for vessels 1-4 respectively. The estimated values for daily production were significantly ($p<0.05$) correlated with the estimates for the amount of Cbl in the washings.

Effects of cobalt supplementation upon fermentation (periods 2 and 3)

Vitamin B12 synthesis For most vessels there were gradual increases in production following each increase in Co input (Figs. 5.4 and 5.5). Mean values for the last 3 sampling occasions in periods 2 and 3 showed increases in Cbl production with all levels of Co supplementation, except the 23.8 nmol Co/d addition into vessel 4 (Table 5.6). Due to the large inter-vessel variation statistical analysis by the paired t-test showed significant increases from the

TABLE 5.7 Experiment 3. Vessel responses (i.e. period 2 or 3 mean minus period 1 mean) in VFA molar proportions (%) and acetate: propionate ratio to cobalt supplementation when barley was given to vessels 1-4 at 7 g DM/d. Mean values were derived from d 18, 21 and 23, d 33, 35 and 37, and d 47, 49 and 51 for periods 1, 2 and 3 respectively.

	Cobalt supplement (nmol/d)	Vessel response				Mean resonse
Molar proportions						
Acetic	11.9	vessel 1)	6.00	2)	3.50	4.75
	23.8	" 3)	3.10	4)	2.10	2.60
	47.6	" 1)	7.10	2)	4.40	5.75
	95.2	" 3)	4.40	4)	5.20	4.80
Propionic	11.9	vessel 1)	-6.30	2)	-9.90	-8.10
	23.8	" 3)	3.70	4)	2.50	3.10
	47.6	" 1)	-3.00	2)	-3.40	-3.20
	95.2	" 3)	-7.80	4)	0.800	4.30
Total butyric	11.9	vessel 1)	-3.90	2)	3.50	-0.20
	23.8	" 3)	-3.90	4)	-8.00	-5.95
	47.6	" 1)	-5.70	2)	-0.60	-3.15
	95.2	" 3)	-6.70	4)	-10.4	-8.55
Total valeric	11.9	vessel 1)	4.53	2)	2.50	3.52
	23.8	" 3)	2.76	4)	2.54	2.65
	47.6	" 1)	1.61	2)	-0.100	0.755
	95.2	" 3)	0.060	4)	2.84	1.45
Acetate: propionate	11.9	vessel 1)	0.422	2)	0.307	0.365
	23.8	" 3)	0.180	4)	-0.055	0.0625
	47.6	" 1)	0.322	2)	0.156	0.239
	95.2	" 3)	0.080	4)	0.090	0.0850

TABLE 5.8 Experiment 3. Vessel responses (i.e. period 2 or 3 mean minus period 1 mean) in VFA production (mmol/d) to cobalt supplementation when barley was given to vessels 1-4 at 7 g DM/d. Mean values were derived from d 18, 21 and 23, d 33, 35 and 37, and d 47, 49 and 51 for periods 1, 2 and 3 respectively.

	Cobalt supplement (nmol/d)	Vessel response				Mean response
Total VFA	11.9	vessel	1)	-3.40	2) -14.5	-8.95
	23.8	"	3)	8.20	4) -1.40	3.40
	47.6	"	1)	-3.20	2) -2.20	-2.70
	95.2	"	3)	2.40	4) -1.20	0.600
Acetic acid	11.9	vessel	1)	1.40	2) -2.60	-0.600
	23.8	"	3)	4.20	4) 0.40	2.30
	47.6	"	1)	1.90	2) -0.700	0.600
	95.2	"	3)	2.60	4) 1.70	2.15
Propionic acid	11.9	vessel	1)	-3.70	2) -9.40	-6.55
	23.8	"	3)	1.70	4) 1.00	1.35
	47.6	"	1)	-2.30	2) -5.20	-3.75
	95.2	"	3)	1.60	4) 0.400	1.00
Total butyric acid	11.9	vessel	1)	-2.53	2) -2.04	-2.29
	23.8	"	3)	0.350	4) -3.59	-1.62
	47.6	"	1)	-3.18	2) -2.36	-2.77
	95.2	"	3)	-2.08	4) -4.47	-3.28
Total valeric acid	11.9	vessel	1)	1.56	2) -0.570	0.495
	23.8	"	3)	1.93	4) 0.890	1.41
	47.6	"	1)	0.400	2) -1.01	-0.305
	95.2	"	3)	0.220	4) 1.04	0.630

unsupplemented level only at the 2 highest levels of Co supplementation. All the vessels exhibited increases in analogue production when supplemented with Co (Table 5.6), but variation was again large and a significant difference (paired t-test, $p<0.05$) from the unsupplemented level occurred at the highest level of supplementation only.

Utilising information from the first 3 periods the level of Cbl production was not correlated with Co input, but the mean responses (i.e. increases over resting values) in Cbl production (y; pmol/d) were significantly related to Co input (x; nmol/d) by the equation:

$$y=5.51x + 24.0 \qquad r=0.973 \qquad d.f.=2.$$

This provides a value for the efficiency for incorporation of the Co supplement into Cbl of 0.551 %.

The corresponding relationship between the response in analogue production (y; pmol/d) and Co input (x; nmol/d) was:

$$y=38.0x + 574 \qquad r=0.999 \qquad d.f=2,$$

i.e. a conversion efficiency value of 3.80 %.

VFA synthesis In view of the vessel differences in Cbl and VFA production, apparent in the responses to Co supplementation (Tables 5.6, 5.7 and 5.8), comparisons between periods were made within vessels. Such analyses (paired t-test) revealed a significant ($p<0.05$) increase in the acetate: propionate ratio at the highest level of Co supplementation. No significant changes occurred in VFA production (Table 5.8), but increasing the Co supplement from 23.8 to 95.2 nmol/d significantly ($p<0.05$) decreased

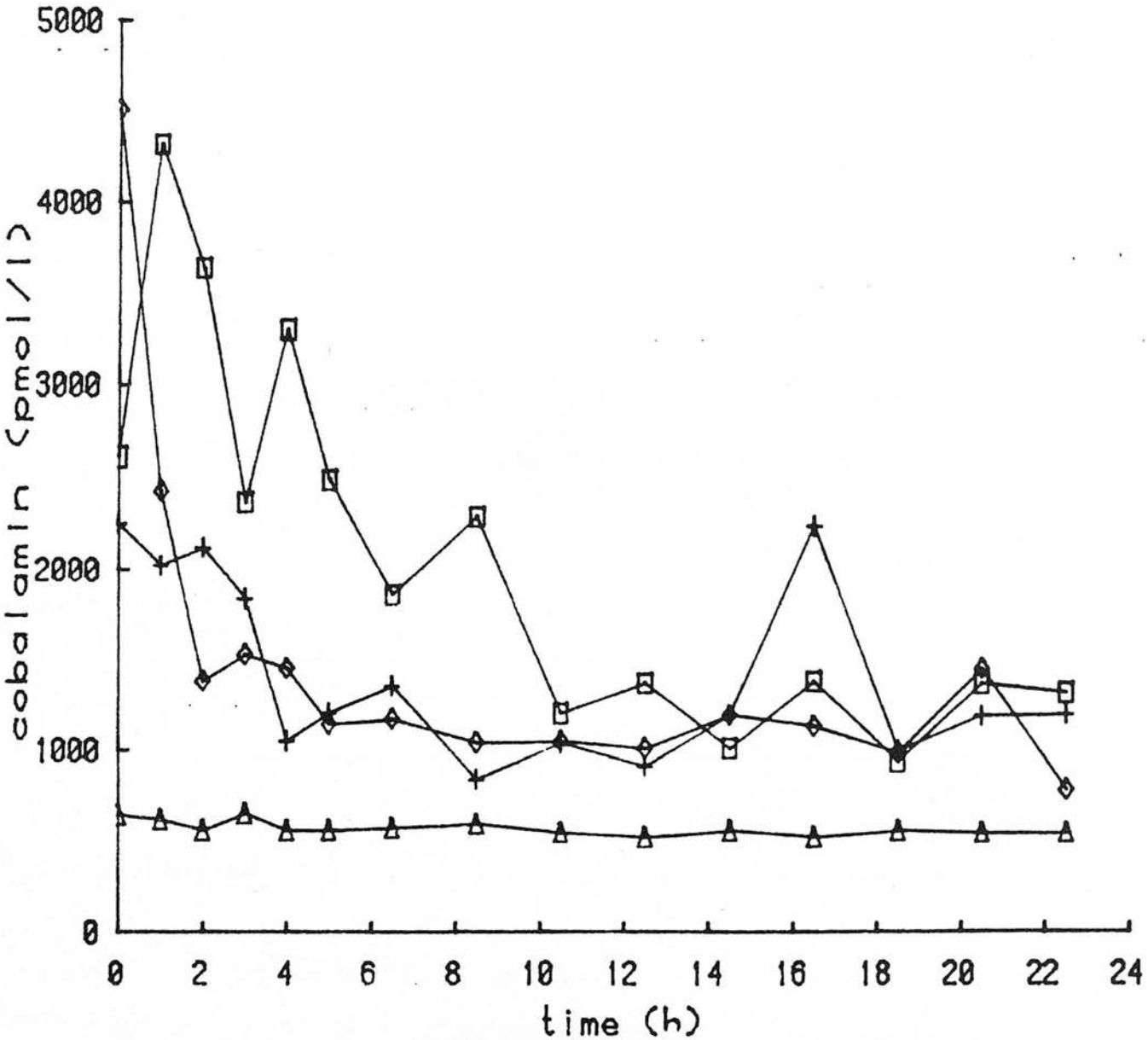


FIG. 5.6 Experiment 3. Diurnal variation in cobalamin levels in compartment 1 of vessels 1-4, given 7 g DM barley/d and supplemented with cobalt, on d 48 (period 3). (supplements: vessels 1 (+) and 2 (△) = 47.6 nmol Co/d, vessels 3 (□) and 4 (◇) = 95.2 nmol Co/d)

the molar proportion of butyric acid; while increasing the supplement from 11.9 to 47.6 nmol Co/d significantly ($p<0.05$) decreased molar proportions of valeric acid and the addition of 23.8 nmol Co/d significantly ($p<0.05$) increased the proportion of valeric acid. The paired t-test is dependent upon agreement between pairs of values and therefore results may appear to conflict. However, the increases in acetate proportions upon supplementation with Co were significant ($p<0.001$) when pooled and analysed by the one sample t-test.

Diurnal variation in cobalamin levels (period 3) There was a stark contrast between the Cbl levels of vessel 2 and those of the other vessels on d 48 (Fig. 5.6). In vessel 2, values were not elevated immediately after feeding and they remained constant throughout the day. The samples from other vessels had high post-feeding values (3-7 times those of vessel 2) that decreased to comparatively constant levels within 10 h and which were twice those of vessel 2.

Estimates of the Cbl contents of the washings were 902, 89.7, 1660 and 2270 pmol for vessels 1-4 respectively. Values for the daily Cbl production, by assessment of the area under the Cbl vs. time curves, were 1,330, 595, 1,810 and 1,260 pmol/d for vessels 1-4 respectively.

Effects of riboflavin supplementation upon fermentation (period 4)

The addition of riboflavin did not have a consistent effect upon Cbl or analogue synthesis, ADMD, VFA production or VFA proportions, for either the unsupplemented or Co supplemented barley (Apps. 3.1, 3.2 and 3.3).

TABLE 5.9 Experiment 3. Mean production values (pmol/d) for cobalamin and analogues during periods 3 (d 47, 49 and 51) and 4 (d 55, 56 and 57), for vessels 1-4 of Rusitec. Supplements were,

Period 3: vessels 1 and 2 = 47.6 nmol Co/d, vessels 3 and 4 = 95.2 nmol Co/d.

Period 4: vessels 3 and 4 = 95.2 nmol Co/d, vessels 1 and 4 = 2.66 umol Riboflavin/d.

		Vessel			
		1	2	3	4
	Period				
Cobalamin	3	918	307	1640	1260
	4	749	330	2140	1320
Analogues	3	3780	2270	5650	5070
	4	645	392	3350	2410

Vitamin B12 synthesis The effect of Co withdrawal on Cbl production was negligible (Fig. 5.5), but it did cause reductions of 82.9 and 82.7 % in analogue production from vessels 1 and 2 respectively, over the 6 d of period 4 (App. 3.1). The continuation of Co supplementation (95.2 nmol/d) in vessels 3 and 4 maintained high levels of Cbl production, but analogue production fell by 40.7 and 52.5 % respectively (Table 5.9).

Samples of washings taken on d 51 and d 57 contained 754, 439, 1420, 732 and 357, 516, 1180, 2640 pmol Cbl in vessels 1-4 respectively. These values (y) correlated with daily Cbl production in the vessels (x) according to the regression equation:

$$y=0.490x + 260 \qquad r=0.860 \qquad d.f.=5$$

The effluent sample from vessel 4 on d 57 was lost.

Discussion

General characteristics of the barley fermentation

The fermentation patterns of the 4 cultures in period 1 were representative of a concentrate diet and the acetate: propionate ratio was similar to that recorded by Orskov *et al.* (1974) when feeding pelleted, ground barley to lambs, although the acetate and propionate proportions in Rusitec were lower and the butyrate and valerate proportions higher than the *in vivo* results. The only previous reported use of barley in Rusitec was as 60 % of a diet with hay that produced molar proportions of 57.2, 23.0, 14.5 and 5.37 % for VFA C2-C5, respectively (Czerkawski and Breckenridge, 1979a), compared to 31.9, 31.0, 27.7 and 7.92 % respectively, in period 1.

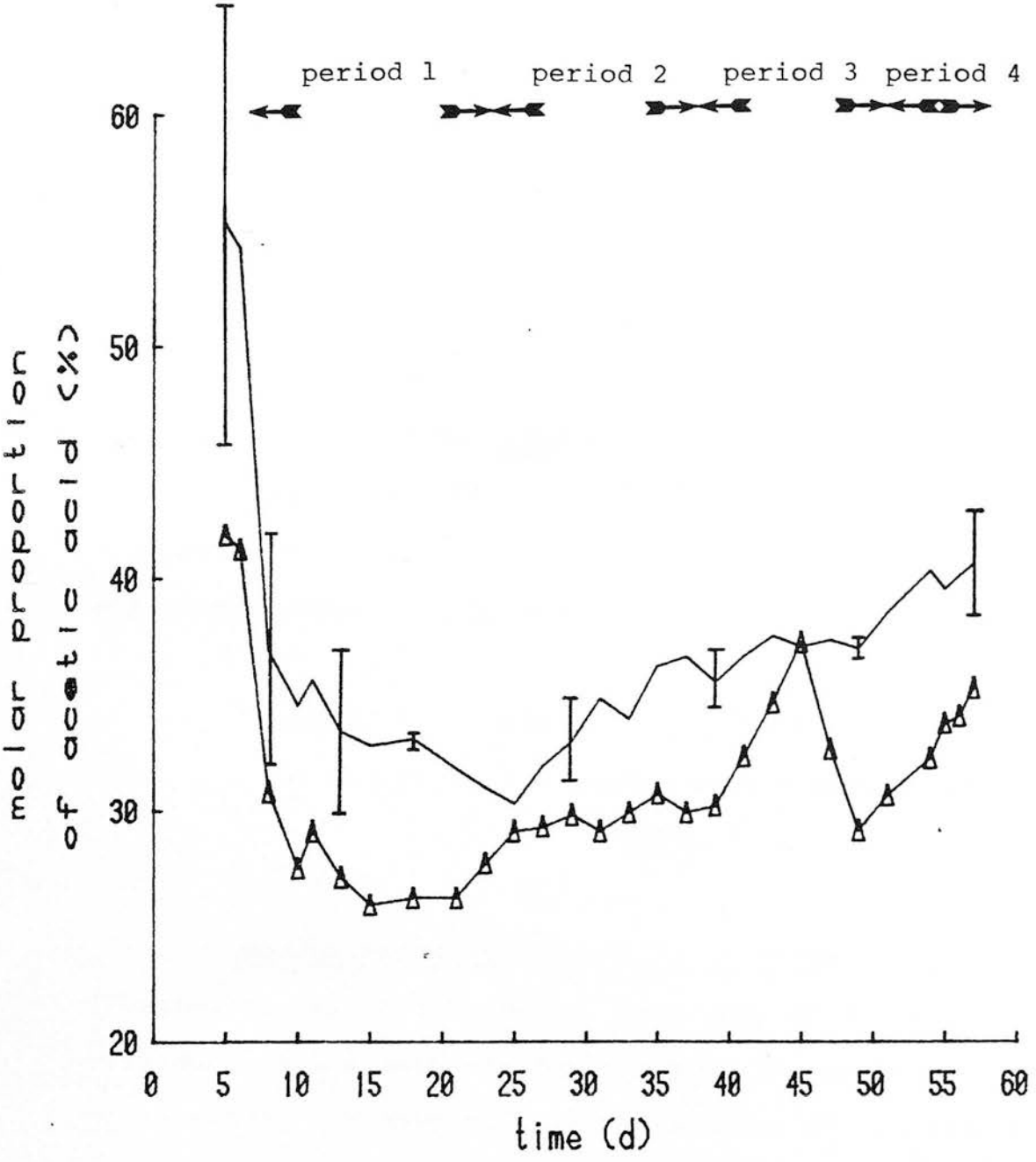


FIG. 5.7 Experiment 3. Molar proportions of acetic acid (%) in total VFA in effluent of vessels 1, 3 and 4 (mean ± s.d.) and vessel 2 (△) during all periods of Experiment 3 when all vessels given 7 g DM barley/d. See Table 5.1 for details of treatments.

ADMD values for the barley were approximately 10 % higher than those for either the pelleted or rolled barley of Orskov et al. (1974), due possibly to the long time that they were retained in Rusitec (48 h). It had been necessary to roll the barley and expose the endosperm as Orskov et al. (1978) found that only 5 % of whole grains were digested when incubated in the rumen. Total VFA produced /g DM digested was found to be similar for both the hay and barley, indicating that the barley had been fermented rather than dissolved.

Vessel differences in fermentation

The differences which arose between vessels given the same food substrate were of 2 types; those which persisted throughout the 57 d experiment and those which changed during the experiment.

Vessel 2 had an atypical fermentation throughout the experiment when compared to the other vessels and this was evidenced by a lower level of Cbl production (Figs. 5.1, 5.4, 5.5), a higher molar proportion of propionic acid and a lower proportion of acetic acid (Fig. 5.7). However, the responses in Cbl production to Co supplementation in periods 2 and 3 were similar to those of vessel 1, which was given the same treatment (Table 5.6). Moreover, the responses in analogue production to Co supplementation in vessel 2 were consistent with those in the other vessels (Table 5.6).

Of interest was the slightly lower ADMD of vessel 2, when fed hay, as the vessel later developed a persistent,

atypical fermentation, while the ADMD of hay in vessel 3 was higher and similar to that in vessels 1 and 4 (Table 5.5).

The fermentation in vessel 3 was initially like that of vessel 2 (Figs. 5.1, 5.2), but a distinct change occurred between d 10 and d 18, which was not reversed during the remainder of the experiment. The "switch" of molar proportions, with no change in ADMD or total VFA production, suggested an adjustment of microbial species rather than numbers. The switch may be related to the use of barley, as Orskov (1973) considered rumen fermentations to be unstable when using this feed. Rumen ciliates did not survive when pelleted grain was fed to sheep (Orskov et al., 1974) due possibly to reductions in pH (Orskov, 1973). Reductions in rumen ciliates have been associated with decreases in the molar proportions of both butyric and acetic acid and with increases in the proportion of propionic acid for cattle fed barley (McDonald et al., 1977). Although pH values were kept constant (ca. pH 7.00) by the infusion of a strong buffer, rumen ciliate survival may have been prejudiced by the low matrix volume provided by the highly digestible barley. The volume of compartment 2 varied from 5-20 ml compared to a mean volume of 30 ml in Experiment 2, when hay was fed. Czerkawski and Breckenridge (1979a) found that the fermentation of hay in Rusitec could also alter noticeably during treatments and perhaps such switches can occur on many diets.

An atypical fermentation had also been encountered in vessel 2 during Experiment 1, but structural differences

between vessels could not be blamed because the vessels were dismantled between experiments and randomly reassembled.

Microbial species differ in their metabolic pathways and changes in species are therefore likely to change fermentation patterns. Microbial synthesis of propionic acid can occur via formation of acrylate or succinate intermediates. The contribution by the acrylate pathway has been found to increase from 0-30 % as the carbohydrate availability of the diet increases, e.g. with a change from a roughage to concentrate (Baldwin et al., 1963). Such changes have been considered to be due to different microbial populations (Whanger and Matrone, 1967) and they may occur even when the same substrate is used in all cultures.

The acrylate pathway is independent of vitamin B12, but the vitamin might be involved in the succinate pathway by virtue of its role as a coenzyme in the migration of a thiol ester group (-COSCoA) in the conversion of succinyl CoA to methylmalonyl CoA, i.e. the reverse of the reaction in propionate metabolism (Fig. 1.3). Thus, the levels of vitamin B12 present in a culture might be determined by the relative importance of the succinate and acrylate pathways. Alternatively, the amount of Cbl present may have determined the nature of the fermentation established. In period 1, the molar proportion of propionate in the effluent was negatively correlated ($p<0.001$) with Cbl production, between all 4 vessels. Cbl may encourage acetate production at the expense of propionate.

Of particular interest were the temporal differences between the switches in Cbl and propionate production in vessel 3. Propionic acid, expressed as a molar proportion of the total VFA, reached its lowest value by d 15 (Fig. 5.2), yet the peak value for Cbl production occurred on d 18 (Fig. 5.1). This difference might have been an indication of the mean time between intracellular Cbl synthesis and its entry into compartment 1. If the liberation of Cbl into compartment 1 occurred immediately after death and lysis of the bacteria within the feed matrix, then the time delay would be shorter. Therefore, it appears that "trapping" (by recycling) of Cbl between microbes delayed the flow between compartments.

Resting values (period 1)

With the exception of vessel 3, Cbl production did not alter substantially with the change in diet, with the result that all the efficiencies of incorporation of Co into Cbl were higher for the barley diet than for the hay (Table 5.3). This is contrary to the general idea that a concentrate diet produces less Cbl than a roughage diet.

The high conversion efficiency (>100 %) of Co into analogues in vessel 3 (Table 5.3) suggested that some values for analogue production in period 1 were possibly overestimates due to carryover effects; as occurred in the initial period of this experiment and in earlier experiments. These carryover effects could have been related to the storage capacity of the microbes in compartments 2 and 3, for forms of vitamin B12 released

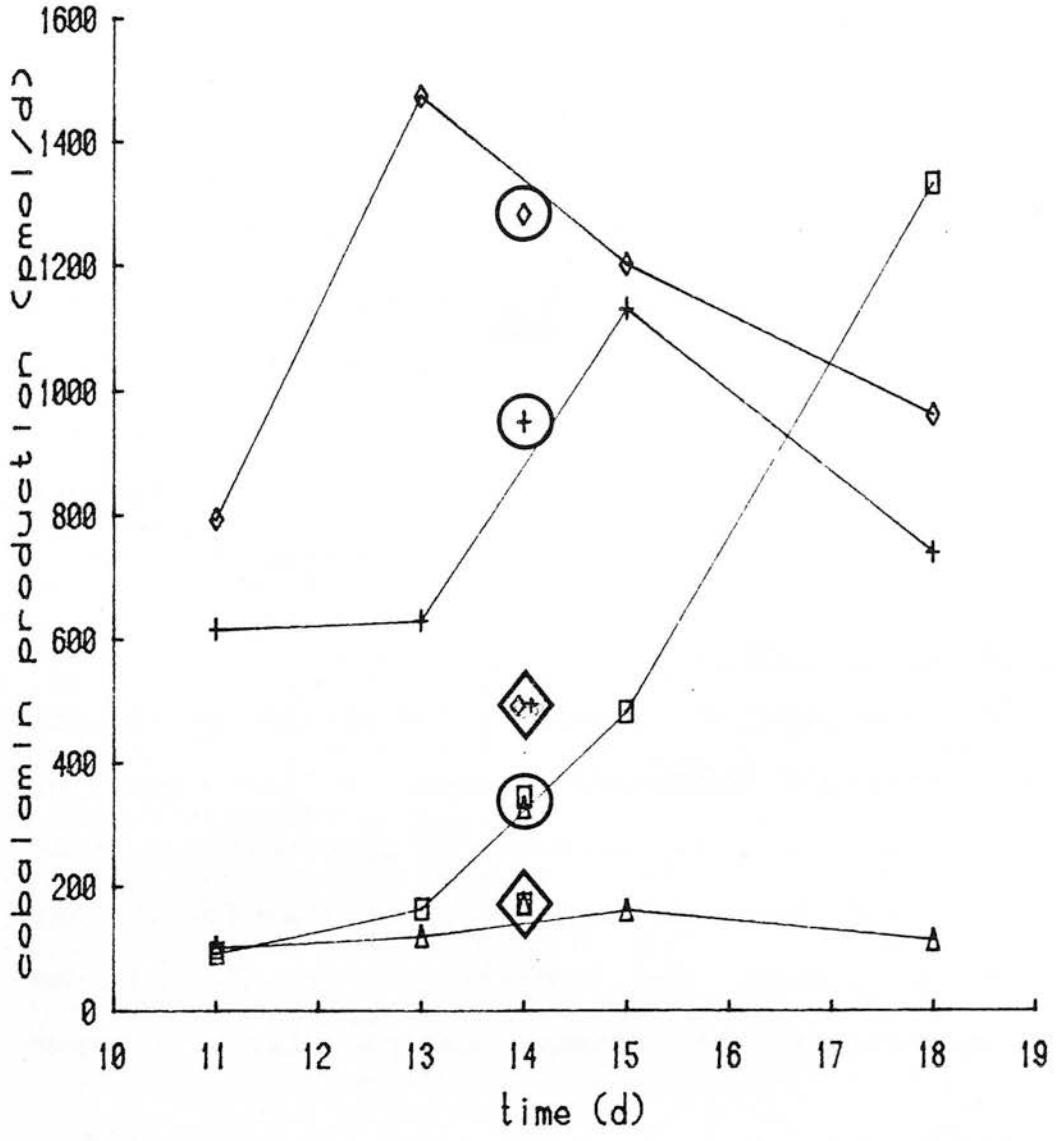


FIG. 5.8 Experiment 3. Cobalamin production values from vessels 1-4 on d 11, 13, 15 and 18 compared with production values on d 14 calculated from the diurnal samples by;
a) the area method (encased in circle),
b) extrapolation of the stable diurnal values to 24 h and determination of an output value (encased in diamond).

from lysed cells. If released analogues competitively inhibited the adsorption of Cbl then "reservoirs" of these analogues may be retained in Rusitec longer than Cbl, and the latter would achieve a steady state more rapidly. Two common rumen analogues, (adenyl) cobamide (Pseudovitamin B12) and (2-methyladenyl) cobamide (Factor A), have been shown to inhibit Cbl uptake in the alga P.malhamensis, even though the analogues are not metabolically active in this organism (Ford, 1958).

Diurnal variation in cobalamin levels (period 1) The stability of Cbl concentrations in all 4 vessels between 12-24 h on d 14 (Fig. 5.3) did not reflect the variation in daily production values that was occurring at this time (Fig. 5.8). Estimates of daily Cbl production (by area) for d 14 were in close agreement, excepting vessel 2, with the values for daily production determined by sampling the effluent (Fig. 5.8) and were better than output values determined by using the value for Cbl in compartment 1 at 24 h. This substantiated the use of the effluent as a measure of productivity and supported the earlier suggestion that the previous estimates based on the sampling of vessel fluid at 24 h should be corrected (Ch. 8, p 208).

The high "area" value for Cbl production from vessel 2 raises the possibility that on d 14 the culture was briefly functioning in a similar way to vessel 3 and that the possibility also existed for this fermentation to "switch". However, a switch did not occur and the reduced level of Cbl production persisted.

Estimated values for the Cbl contents of the washings for all the vessels were of the same order as values for daily Cbl production on d 13 and d 15. This emphasised the continuing importance of compartment 2 as a source of Cbl, despite its small volume (5-20 ml) when a highly digestible substrate, such as barley, was used.

Effects of cobalt supplementation upon fermentation (periods 2 and 3)

As the values for analogue production in period 3 had achieved stability, this allows for comparison with the results of Elliot et al. (1971), who fed sheep a Co-supplemented barley diet. Their dietary Co concentration of 29.5 µmol Co/kg DM sustained the daily production of Cbl and analogues at 574 and 2410 pmol/g DM respectively, compared to 207 and 766 pmol/g DM for the highest level of supplementation (13.8 µmol Co/kg DM) in Rusitec. Conversion efficiencies, in vivo, were 2.43 and 10.2 % for Cbl and analogue synthesis respectively, and 0.551 and 3.80 % respectively, for inorganic Co in Rusitec.

The linear regression lines for Cbl and analogue responses to Co input indicated that the proportion of Cbl synthesised in total vitamin B12 did not alter with increased Co input; this contrasts with the widely held view that a decrease occurs (e.g. Gawthorne, 1970a; Smith and Marston, 1970a).

Although a few significant differences in VFA production were found within vessels, using the paired t-test, the lack of a consistent trend over all levels of Co

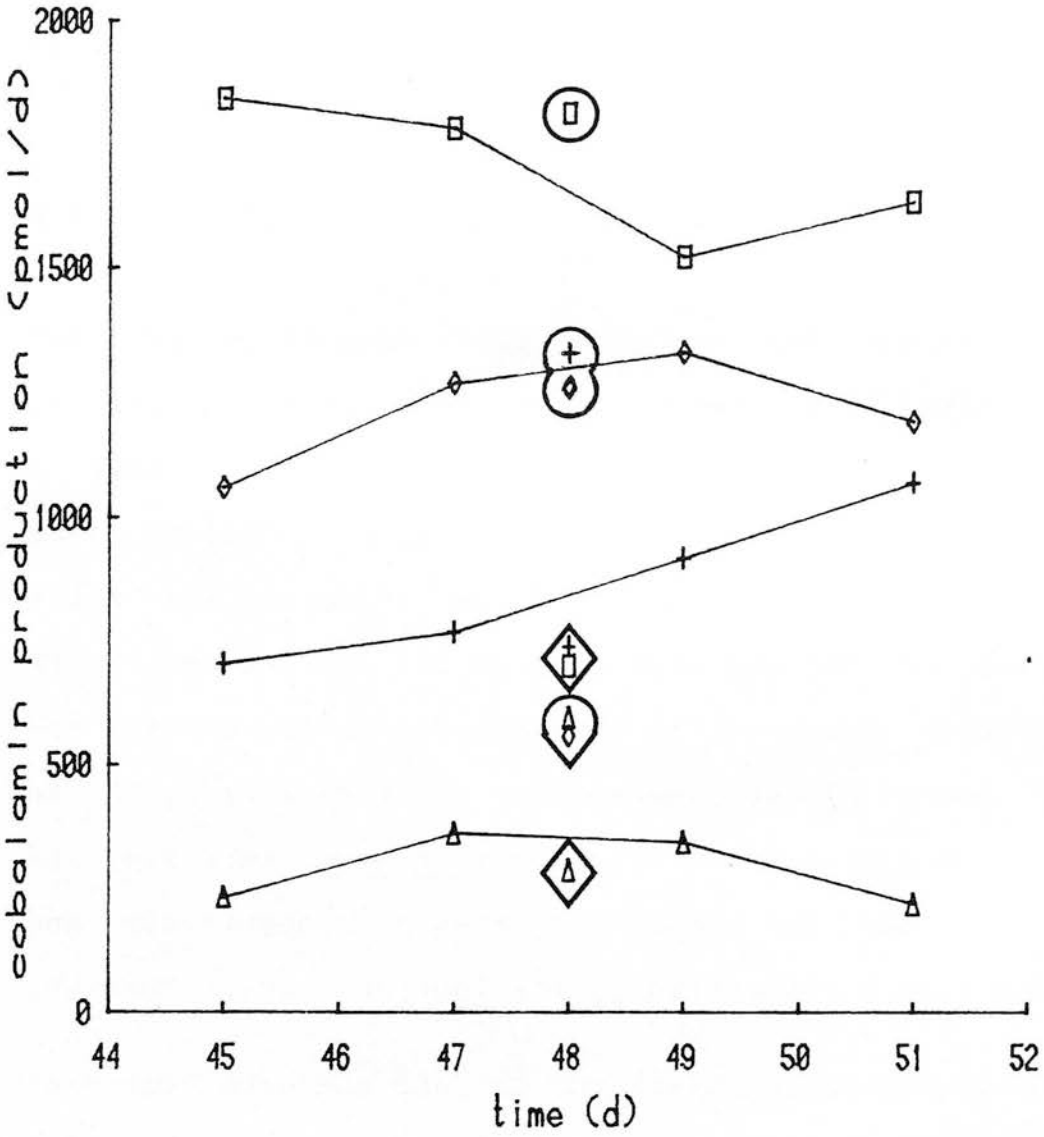


FIG. 5.9 Experiment 3. Cobalamin production values from vessels 1-4 on d 45, 47, 49 and 50 compared with production values on d 48 calculated from the diurnal samples by;
a) the area method (encased in circle),
b) extrapolation of the stable diurnal values to 24 h and determination of an output value (encased in diamond).

supplementation suggested that the differences may have been fortuitous. There was, however, a significantly higher proportion of acetate produced from a given vessel when Co was infused (mean response of 4.48 %, Table 5.7).

Diurnal variation in cobalamin levels (period 3) After reintroduction of the washings, Cbl levels were not expected to increase, as they did in vessel 3, after 1 h (Fig. 5.6). This increase could have been a result of sampling before complete mixing had occurred. Instability was evident in all the vessels, except vessel 2, throughout the 24 h and may be attributable to the Co supplement being supplied to compartment 1. Co supplements provided directly to compartment 1 may have a rapid, "unbuffered" effect on Cbl synthesis in this compartment (the possibility of a buffering ability in certain compartments was discussed on p 120) and/ or they may stimulate the migration of vitamin B12-synthesising microbes from compartment 2.

Figures for daily Cbl production, derived from diurnal Cbl levels, were in close agreement with effluent productivity values for vessels 3 and 4 (Fig. 5.9). The values for vessels 1 and 2 were high, but may reflect true inter-day variation.

The estimated value of 1,660 pmol for the Cbl content of the washings from vessel 3 on d 48 can be compared with the direct measurements of 1420 and 1180 pmol Cbl on d 51 and d 57 respectively. Compartment 2 in vessel 3 may have had a declining Cbl content during this period of Co

supplementation (95.2 nmol Co/d), possibly as a result of migration of microbes and associated vitamin B12 into compartment 1. The production estimates were, with the exception of vessel 2, noticeably greater than the values for output at 24 h (Fig. 5.9).

Effects of cobalt withdrawal upon vitamin B12 production (period 4)

The dilution effects of infusing Co-free artificial saliva would account for a 67.5 % reduction in both Co and vitamin B12 levels /d in compartment 1. However, levels of Cbl production were not greatly reduced by withdrawal of the Co supplement after 6 d, although analogue production fell substantially (App. 3.1). Previously, Cbl production had been the more sensitive indicator of Co supply. The maintenance of Cbl production in the days following Co withdrawal can only be explained satisfactorily by a compensatory flow from compartment 2 and it would appear that the flow of analogues was less marked.

If the values for analogue production in periods 1 and 4 are compared in vessels 1 and 2 it appears that the carryover effect of the original hay diet (543 nmol Co/kg DM) upon period 1 was greater than that of Co supplementation (period 3, 13,700 nmol Co/kg DM) upon period 4, even though the time allowed for equilibration was twice as long in period 1 (12 d vs. 6 d). This difference may be explained by a shift in the distribution of analogue from compartments 2 to 1 during Co supplementation. Consequently, there would be a reduced, inhibitory effect on Cbl uptake in compartment 2 by the

analogues and a more rapid dilution of analogue concentration. Microbial storage (retention) of Co, during the previous period of supplementation, might also have contributed to Cbl synthesis during period 4. Intracellular Cbl synthesis might be less susceptible to analogue inhibition than adsorption.

The Cbl contents of the washings from vessel 2 on d 51 and d 57 were greater than the Cbl production for those days, while for the other vessels the smaller contributions were still substantial. It should also be emphasised that the Cbl content of the washings in the bag of fully (48 h) digested feed is probably more representative of the "24 h" bag for a readily digested substrate such as barley than for a hay.

Effects of riboflavin supplementation upon vitamin B12 synthesis (period 4)

Even though cereals are a poor source of riboflavin, supplementation with this vitamin did not elevate Cbl production at high or low Co inputs. This could be because;

1) riboflavin was non-limiting,

2) insufficient riboflavin was added,

3) the response in Cbl production was not reflected in the effluent because of the short duration of the treatment.

As far as the second possibility is concerned the riboflavin added daily was theoretically sufficient to produce enough 5,6 dimethylbenzimidazole for more than 1000 times the daily Cbl production achieved. Although the distribution of added riboflavin may not have been uniform

throughout the vessel, and a considerable dilution of the riboflavin concentration in compartment 1 would have been expected during the 24 h following supplementation, there should have been sufficient precursor to stimulate Cbl synthesis if it was limiting production. The Cbl content of washings from the riboflavin and Co supplemented vessel on d 57 showed a 360 % increase over the value before riboflavin was added (d 51). This might reflect the delay between synthesis and liberation of Cbl and supports the third postulate. However, vessel 1 which was not supplemented with Co showed a 52.7 % decrease over the same period; therefore, any delayed effect of riboflavin must be dependent upon the Co level. Further investigations with a longer period for equilibration are required but the first postulate provides the most probable explanation.

Conclusions

1. Barley was succesfully employed as a substrate in Rusitec and it produced a "propionate" fermentation. However, differences between cultures were apparent for both the hay and the barley substrate. Conversion efficiencies for Cbl were increased by a change to the barley ration.

2. Analogue synthesis was more susceptible to carryover by compartments 2 and 3 after periods when hay was fed than after Co supplementation of the barley diet.

3. Upon supplementing with Co, conversion efficiencies for both Cbl and analogue synthesis were reduced to values less than those for the same Co supplement to a hay ration (Experiment 2).

4. An association between Co supplementation of the barley and an increase in the proportion of acetate in the total VFA was revealed.

5. The use of the effluent as a measure of productivity gave increased values and confirmed the need for correcting previous output values.

6. Riboflavin was not shown to influence vitamin B12 synthesis, although longer-term studies may be necessary to establish a possible relationship.

CHAPTER 6

EXPERIMENT 4. THE AVAILABILITY OF SOIL COBALT FOR RUMINAL VITAMIN B12 SYNTHESIS

Introduction

Soil, accidentally ingested by herbivores, can be an important source of trace elements (Field and Purves, 1964; Healy *et al.*, 1974). As long ago as 1934 Rigg and Askew showed that drenching sheep with a suspension of Nelson soil was efficacious in remedying bush-sickness, whereas Onekaka limonite was not. More recently, Macpherson *et al.* (1978) found that soils differed in their ability to replete Co-deficient sheep and that this faculty was dependent upon factors other than Co content. They suggested that soils should not be considered solely in terms of the Co status of the plants that grew on them.

An attempt was therefore made to obtain quantitative information on the extent to which the Co from 4 diverse soils was available for vitamin B12 production in Rusitec cultures compared with the form of Co supplementation (Co nitrate) investigated previously.

Experimental procedure

The inoculum for Rusitec was pooled rumen samples from fistulated Blackface x Suffolk sheep fed on Ruminant A diet, the stock of low Co hay(1) used previously being insufficient to feed donor animals. A mixed grass hay(2), different from that used in Experiments 1, 2 and 3 (Table

TABLE 6.1 Experiment 4. Treatments used to investigate the effects of soil type and inclusion rate on the synthesis of vitamin B12 by 4 Rusitec cultures given low cobalt hay(2) at 7.22 g DM/d and supplemented with soil.

		Vessel			
		1	2	3	4
Period	Days				
1	1-8	<----------no supplement---------->			
2 (0.7 g soil/d)	9-22	chalk	sand	clay	weald loam
3	23-27	<----no supplement & "cleaning"----->			
4 (1.4 g soil/d)	28-37	-	chalk	weald loam	-
5	38-43	<----------no supplement---------->			

TABLE 6.2 Experiment 4. Characteristics of soils used as sources of Co in Rusitec (µmol/kg DM unless stated).

Soil	OM (g/kg DM)	Iron (mmol/kg DM)	Copper	Zinc	Manganese	Cobalt	
						Total	Acetate-extr.
Chalk	163	327	221	1470	1690	365	1.48*
Sand	13.5	25.1	58.3	19.9	4.00	11.9*	0.144*
Clay	74.4	258	96.1	535	1330	1100	27.2
Weald loam	87.3	4220	1310	2980	714	28.9*	9.78

*= deficient with regard to plant growth in total Co (< 84.9 µmol/kg DM) and/or acetate-extractable Co (<5.09 µmol/kg DM) (COSAC, 1982).

3.1), but of low Co content (0.049 mg Co/kg DM = 832 nmol Co/kg DM), was used as the food substrate at 7.22 g DM/d (period 1, d 1-8).

The treatments employed are outlined in Table 6.1. After 8 d, 4 different soils, a chalk, a sand, a clay and a weald loam were added to vessels 1-4 respectively at 0.7 g DM/d, as supplements to the low Co hay(2). These soils differed greatly in their Co content, as well as in OM, manganese, iron, copper and zinc contents (Table 6.2); these latter 3 were in the greatest concentration in the weald loam and decreased in the order chalk> clay> sand. The release of the elements from the soils in Rusitec was also investigated. The soils were added directly to the vessel liquid (compartment 1) when the food was replenished for the following 14 d (period 2, d 9-22). After this, the vessels were then "cleaned" of residual soil particles by decanting off most of compartment 1 and removing the solid, which was dried at 100 °C and analysed for acetate-extractable and total Co. The unsupplemented hay was continued for 5 d (period 3, d 23-27) in order to further cleanse the system and deplete the cultures of vitamin B12. During this period (d 25) the culture in vessel 2 was contaminated by liquid from the waterbath; therefore, the vessel fluid was discarded and replaced by mixing the fluid compartments from the other vessels and dispensing the volume equally between the 4 vessels.

The soils producing the greatest response in Cbl production during period 2, the chalk and the weald loam, were used for a second period of supplementation, at

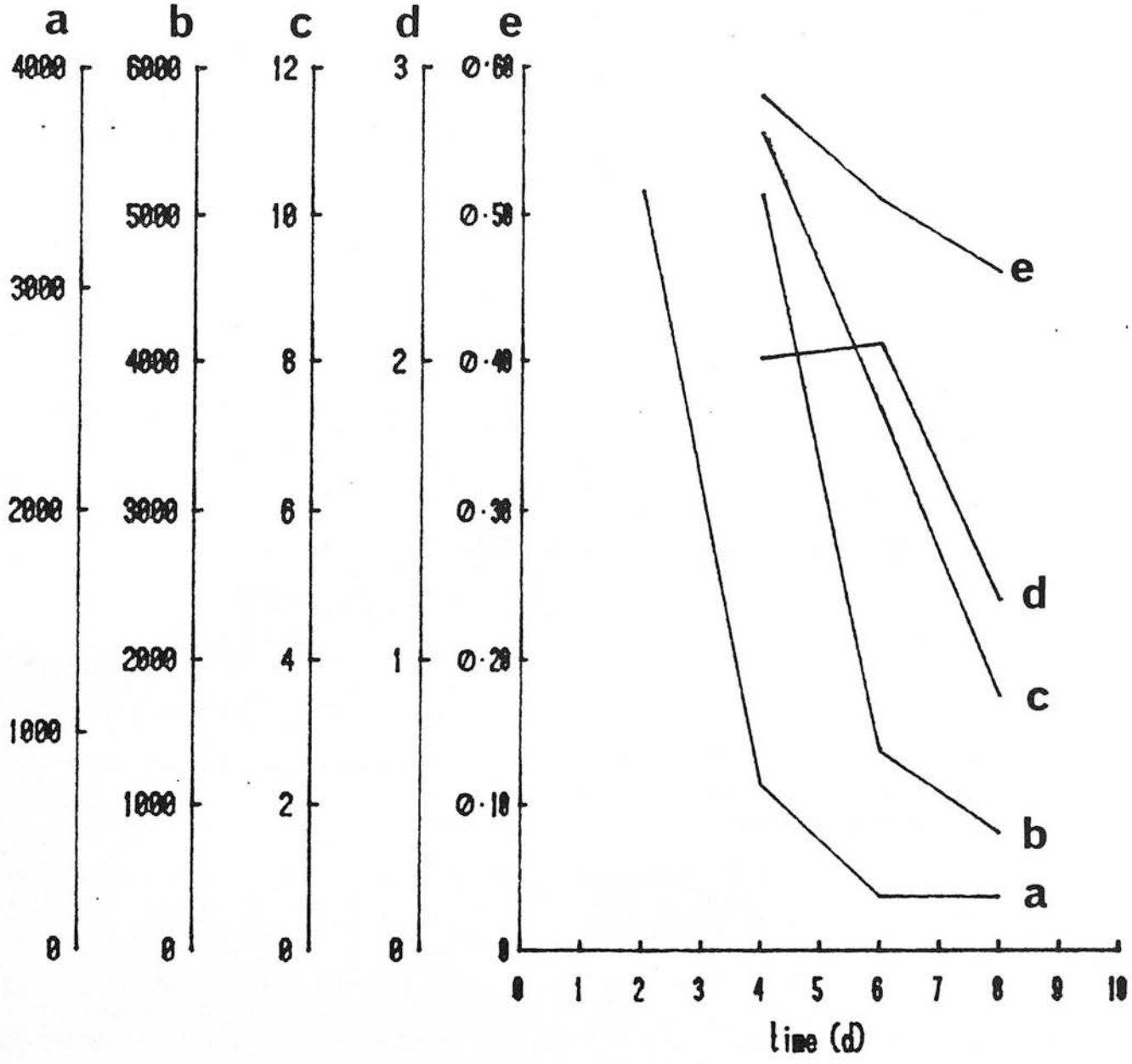


FIG. 6.1 Experiment 4. Stabilisation of vitamin B12 production values and recoveries of elemental analytes when vessels 1-4 were given 7.22 g DM low cobalt hay(2)/d. Value for each day is mean of all four cultures. The analytes were:

a = cobalamin production (pmol/d)

b = analogue production (pmol/d)

c = iron recovery (μmol/d)

d = zinc recovery (μmol/d)

e = copper recovery (μmol/d).

TABLE 6.3 Experiment 4. Cobalamin (d 6 and d 8) and analogue (d 8) production (pmol/d), the respective conversion efficiencies (%) and the cobalamin content of the washings (pmol) on d 8 during period 1, when the cultures in vessels 1-4 were given low cobalt hay(2) at 7.22 g DM/d.

	Vessels				
	1	2	3	4	mean ± s.d.(n=4)
Production					
Cobalamin	551	148	113	175	247 ± 204
Analogues	1320	59.0	383	484	562 ± 537
Conversion efficiencies					
Cobalamin	9.21	2.48	1.89	2.93	4.13 ± 3.42
Analogues	22.1	0.987	6.41	8.09	9.40 ± 9.00
Cobalamin in washings	990	89.0	81.7	447	402 ± 428

1.4 g DM/d for 10 d, in vessels 2 and 3 respectively. Vessels 1 and 4, which had received those soils in period 2 were left unsupplemented (period 4, d 28-37). At the end of this period the residual soil from vessels 2 and 3 was salvaged, dried and analysed as before. With the cessation of soil supplementation, all the vessels continued to receive low Co hay(2) for a further 6 d (period 5, d 38-43).

As in Experiment 3, the production of Cbl, analogues and VFA by the cultures was measured by sampling the effluent; in addition the effluent was assayed for iron, copper and zinc (p 96), in order to determine if the release of these elements from the soils was related to the availability of Co. Samples of the washings were taken throughout the experiment and analysed for Cbl. The iron, copper and zinc contents of the hay used were 2,300, 92.7 and 147 µmol/kg DM respectively.

Results

Resting values with unsupplemented hay (period 1)

Cbl and analogue production declined rapidly in period 1 (Fig. 6.1) and equilibrium appeared to have been reached by d 6 for Cbl but not for analogue production (Fig. 6.1, App. 4.1). Therefore, d 6 and d 8 were used to determine mean production for Cbl and the value for d 8 was taken as the best estimate of analogue production (Table 6.3). Cbl and analogue production were higher from vessel 1 than from the other vessels and vessel 2 produced a noticeably low level of analogues on d 8. Consequently, the coefficients

TABLE 6.4 Experiment 4. VFA molar proportions (%) and the acetate: propionate ratio in period 1 for the cultures in vessels 1-4, given 7.22 g DM low cobalt hay(2)/d. Values are means of d 4, 6 and 8.

	Vessel			
	1	2	3	4
Proportions				
Acetic acid	58.4	49.0	48.3	50.4
Propionic acid	21.3	27.4	27.1	25.6
Total butyric acid	12.9	14.0	13.5	13.9
Total valeric acid	7.24	9.64	10.7	9.93
Acetate: propionate	2.77	1.80	1.79	1.98

of variation between vessels for Cbl (82.6 %) and analogue (95.6 %) production were large. Conversion efficiencies for the synthesis of these compounds ranged from 1.89-9.21 % for Cbl and 0.987-22.1 % for analogues (Table 6.3). The Cbl contents of the washings on d 8 were significantly ($p<0.05$) correlated with Cbl production on that day (Table 6.3); for vessels 1 and 4 the amounts were approximately twice the production values, whereas for the other 2 vessels there was less Cbl in the washings than in the effluent.

Recoveries of iron and copper on d 8 (Fig. 6.1), were far less than daily inputs, the ranges being 19-23 % of the total daily input for iron and 64-78 % for copper. Zinc recoveries were not all less than daily inputs, covering the range 88-142 % of the total daily input. Outputs of all 3 elements had not equilibrated by d 8 (Fig. 6.1) but were uniform between vessels, the c.v. for iron, copper and zinc being 7.75, 9.56 and 20.8 % respectively.

The fermentation patterns of all 4 vessels were not similar and the culture in vessel 1 produced proportions of acetic and propionic acid that were respectively higher and lower than the other vessels, giving a 50 % higher acetate: propionate ratio (Table 6.4). Both ADMD and AOMD values for the substrate were higher in vessel 1 (0.586 and 0.594 respectively) than in vessels 2-4 (means ± s.d of 0.571 ± 0.004 and 0.581 ± 0.0045 respectively).

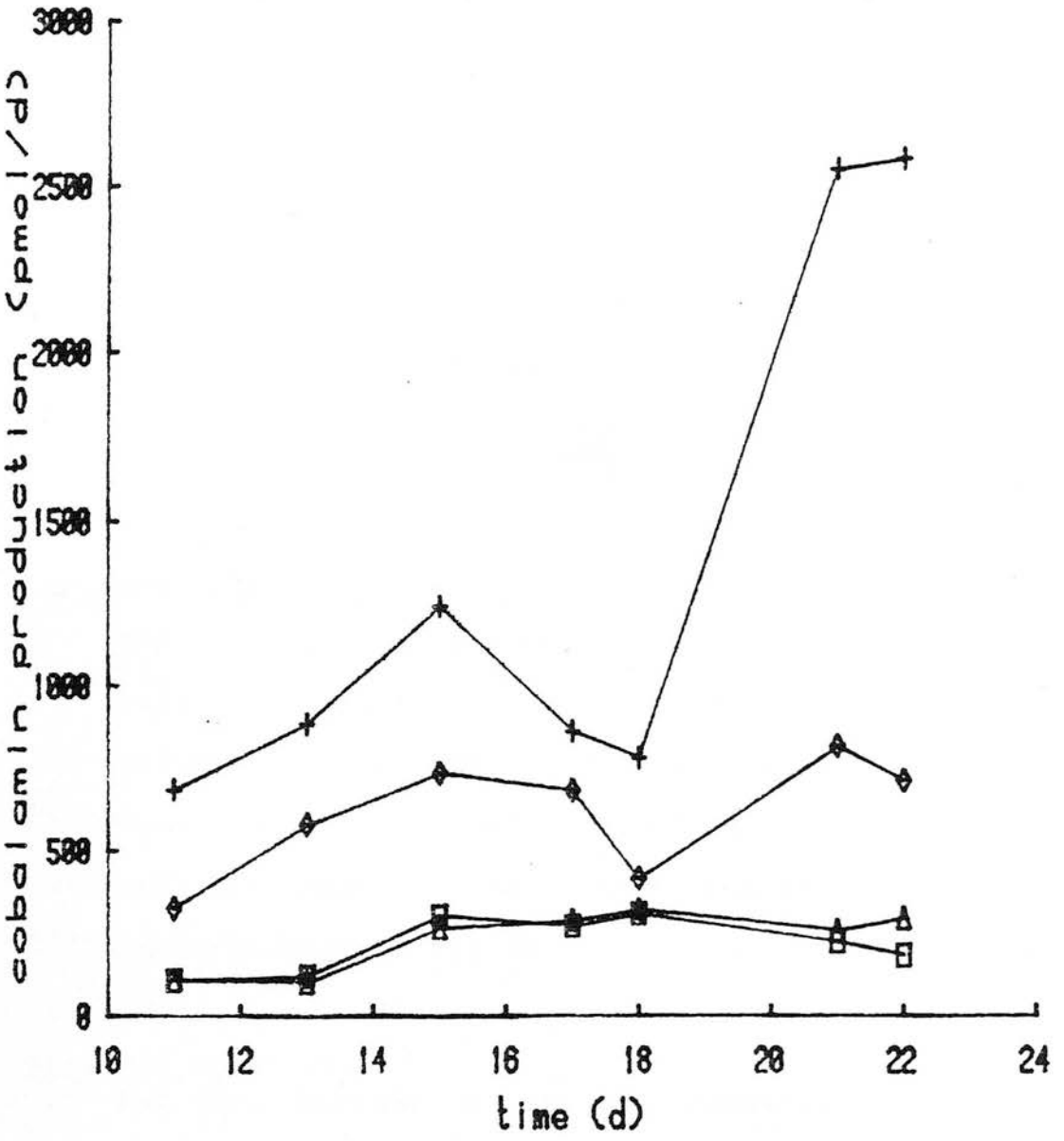


FIG. 6.2 Experiment 4. Cobalamin production (pmol/d) during period 2 for vessels 1-4 given 7.22 g DM low cobalt hay(2)/d and supplemented with one of four soils at 0.7 g/d. (supplements: vessel 1 (+) = chalk, vessel 2 (△) = sand, vessel 3 (□) = clay, vessel 4 (◇) = weald loam)

TABLE 6.5 Experiment 4. Responses (R, mean value of period minus mean value from corresponding vessel during period 1) in cobalamin and analogue production (pmol/d) to soil supplementation at 0.7 and 1.4 g DM/d during periods 2 and 4 respectively. Lower figures denote responses as percentages (P) of the resting values. Details of treatments are given in Table 6.1. Number of days used to derive mean values are in parentheses.

		Soil			
		chalk	sand	clay	weald loam
Cobalamin					
Period 2	R	2010(2)	142(3)	124(3)	586(2)
	P	365	96.0	110	335
Period 4	R	737(3)	-	-	757(2)
	P	493			670
Analogues					
Period 2	R	6770(2)	1860(2)	6980(3)	4760(2)
	P	513	3150	1820	984
Period 4	R	2910(3)	-	-	2820(3)
	P	4930			736

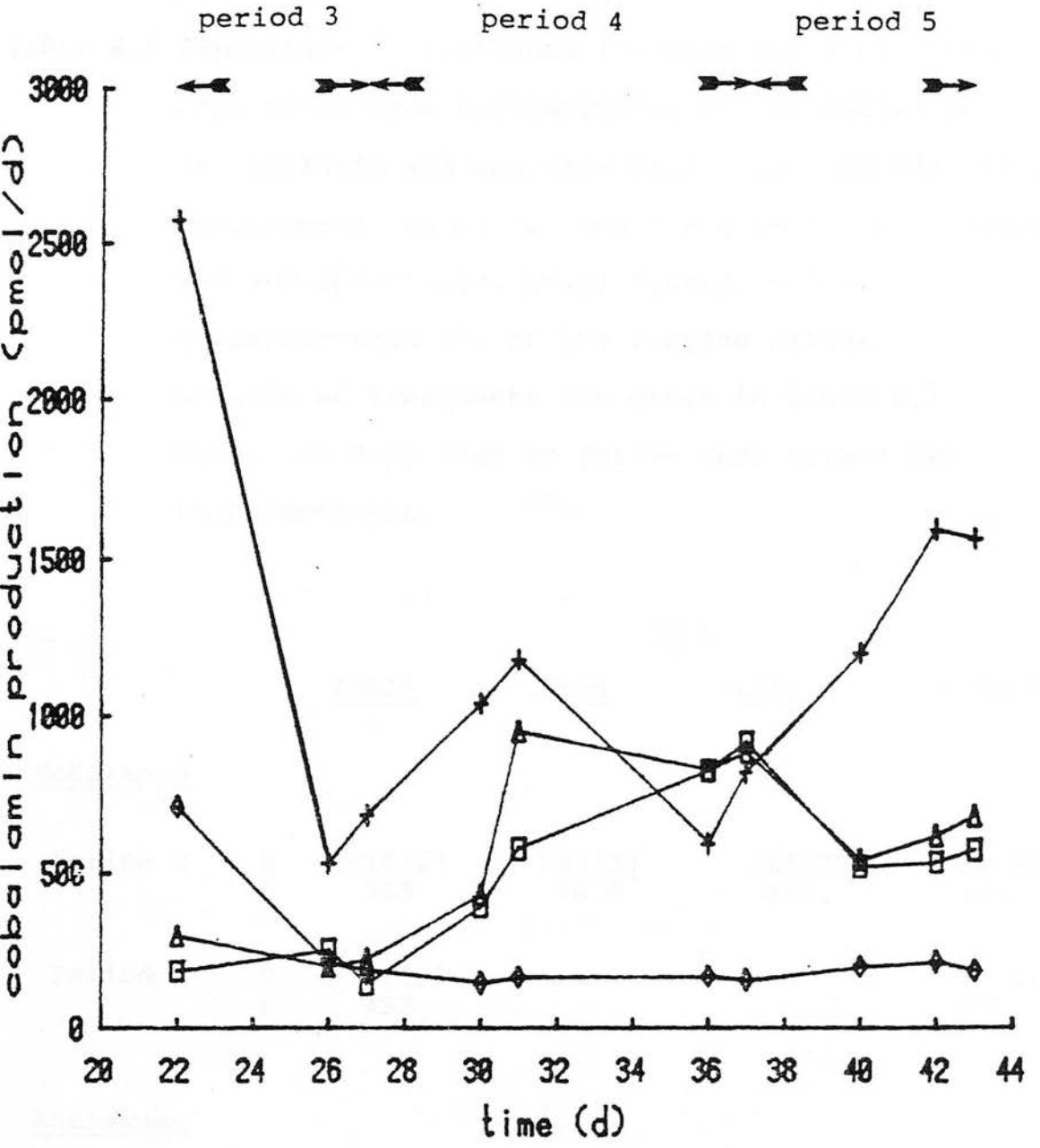


FIG. 6.3 Experiment 4. Cobalamin production (pmol/d) during periods 3, 4 and 5 when all four vessels were given 7.22 g DM low cobalt hay(2)/d. Chalk and weald loam at 1.4 g/d were given as a supplement to vessels 2 (△) and 3 (□) respectively during period 4.

TABLE 6.6 Experiment 4. Conversion efficiencies (%) for total soil cobalt incorporated into cobalamin and analogues during periods 2 (0.7 g soil DM/d) and 4 (1.4 g soil DM/d), using values from TABLES 6.2 and 6.5.

	Soil			
	chalk	sand	clay	weald loam
Cobalamin				
Period 2	0.785	1.71	0.016	2.90
Period 4	0.144	-	-	1.86
Analogues				
Period 2	2.65	22.3	0.907	23.6
Period 4	0.568	-	-	6.98

Effects of soil supplementation upon vitamin B12 synthesis (periods 2 and 4)

Cobalamin and analogue synthesis The addition of 0.7 g/d of each soil gradually increased Cbl production during period 2 (Fig. 6.2), but the increases were not proportional to the Co content of the soils, either total or acetate-extractable; for example, the clay, which had the highest levels of both measures of soil Co, stimulated Cbl production less than the chalk or weald loam, whether expressed in absolute or percentage terms (Table 6.5). The increases in analogue production were far greater than the responses for Cbl in the corresponding vessel and were ranked with, but not linearly related with, total soil Co.

The increased level of soil supplementation in period 4 (1.4 g/d) elevated Cbl production from the levels of period 1 (Fig. 6.3, Table 6.5). The responses were similar for Cbl (737 and 757 pmol) and analogue (2910 and 2820 pmol) production from the chalk and the weald loam respectively and both cultures had a low resting value during period 1 (Tables 6.3 and 6.5). However, only for Cbl production from the weald loam was the level of production increased above the levels of period 2 for the corresponding soil.

The conversion efficiencies for Cbl synthesis, derived from total Co added in the soil (Table 6.6), were all less for the soils than for the unsupplemented hay used in the same vessel during period 1 (Table 6.3) and for the chalk and the weald loam these values decreased further at the higher supplementation level of period 4 (Table 6.6). The

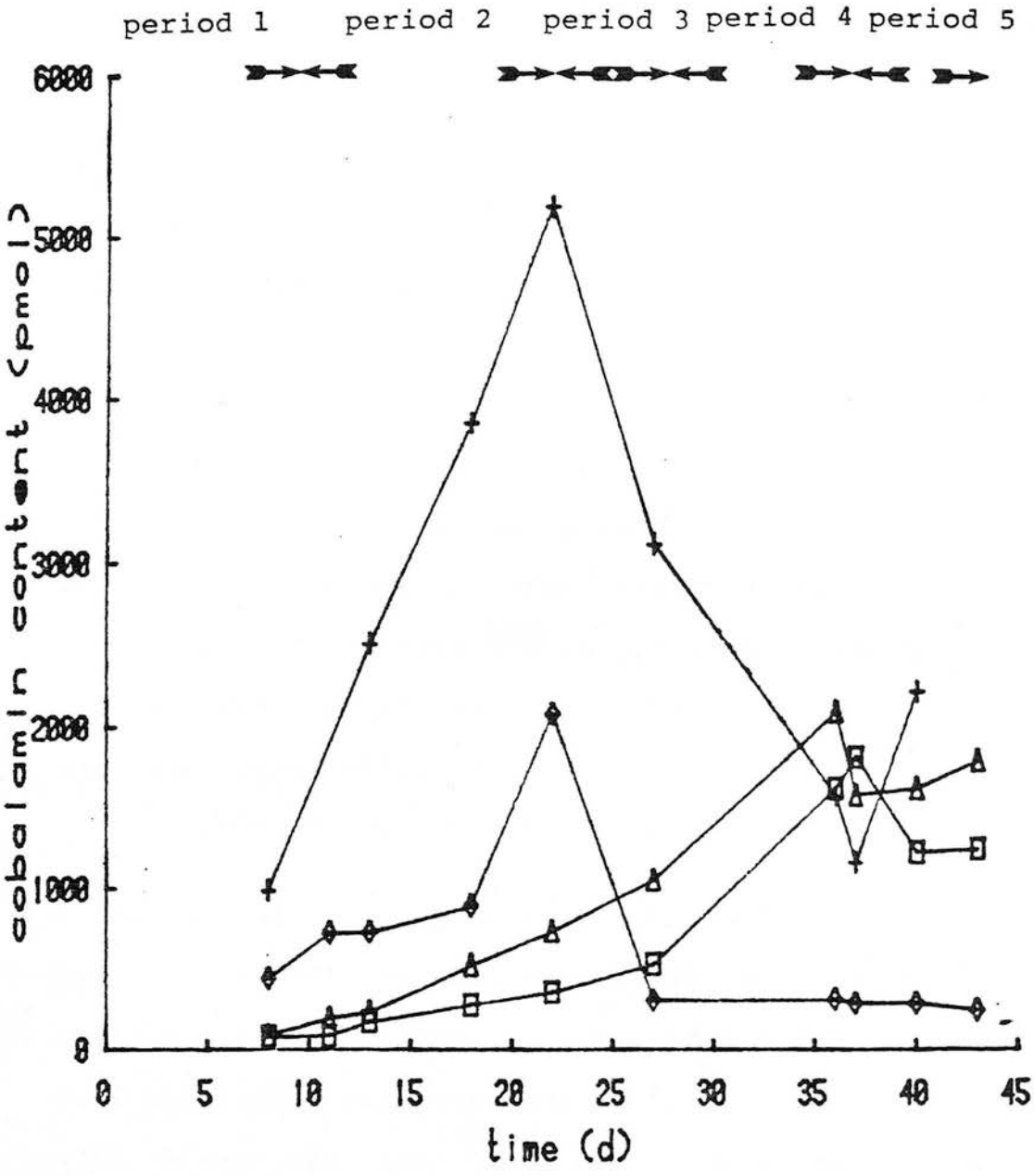


FIG. 6.4 Experiment 4. Cobalamin content (pmol) of the washings from vessels 1-4 given 7.22 g DM low cobalt hay(2)/d during all periods of Experiment 4. Soil supplements given during periods 2 (0.7 g/d) and 4 (1.4 g/d) were as follows:

	Period 2	Period 4
vessel 1 (+)	chalk	---
vessel 2 (△)	sand	chalk
vessel 3 (□)	clay	weald loam
vessel 4 (◇)	weald loam	--- .

efficiencies of analogue synthesis were lower than those of the unsupplemented hay for both the chalk and the clay, but not for the sand and the weald loam during period 2 (Table 6.6). With the 2 soils, chalk and weald loam, added at 2 levels the conversion efficiencies for analogue synthesis decreased with the increased soil input in period 4 (Table 6.6).

Cobalamin content of the washings The Cbl content of the washings showed larger and steadier increases following soil supplementation in period 2 than did Cbl production (Fig. 6.4 vs. 6.2). Furthermore, there was no indication that the amount of Cbl contained in the washings had equilibrated for any soil by d 22 (Fig. 6.4). At the end of period 2 (d 22), the Cbl content of the washings from vessels 1-4 were between 2-3 times those of the effluent and on this day the Cbl content of the washings correlated ($p<0.01$) with Cbl production (Table 6.3), but not with analogue production or either measurement of soil Co.

Supplementation of vessels 2 and 3 with the chalk and the weald loam respectively, at 1.4 g DM/d, increased the rate of accumulation for Cbl in the washings during period 4, although the rates were lower than those achieved by the same soils in vessels 1 and 4 when supplemented at 0.7 g DM/d. Amounts of Cbl in the washings on d 37 were 1.79 and 1.98 times those in the effluent for the chalk and the weald loam respectively.

TABLE 6.7 Experiment 4. Cobalt content (μmol/kg DM) of the soils salvaged from the vessels at the end of supplementation.

Soil	Quantity salvaged (g), after drying at 100 °C	Cobalt	
		Total	Acetate-extr.
Period 2			
Chalk	5.3	125	10.1
Sand	6.0	225	0.866
Clay	7.0	738	43.2
Weald loam	6.0	154	6.01
Period 4			
Chalk	5.5	221	8.25
Weald loam	8.0	178	4.58

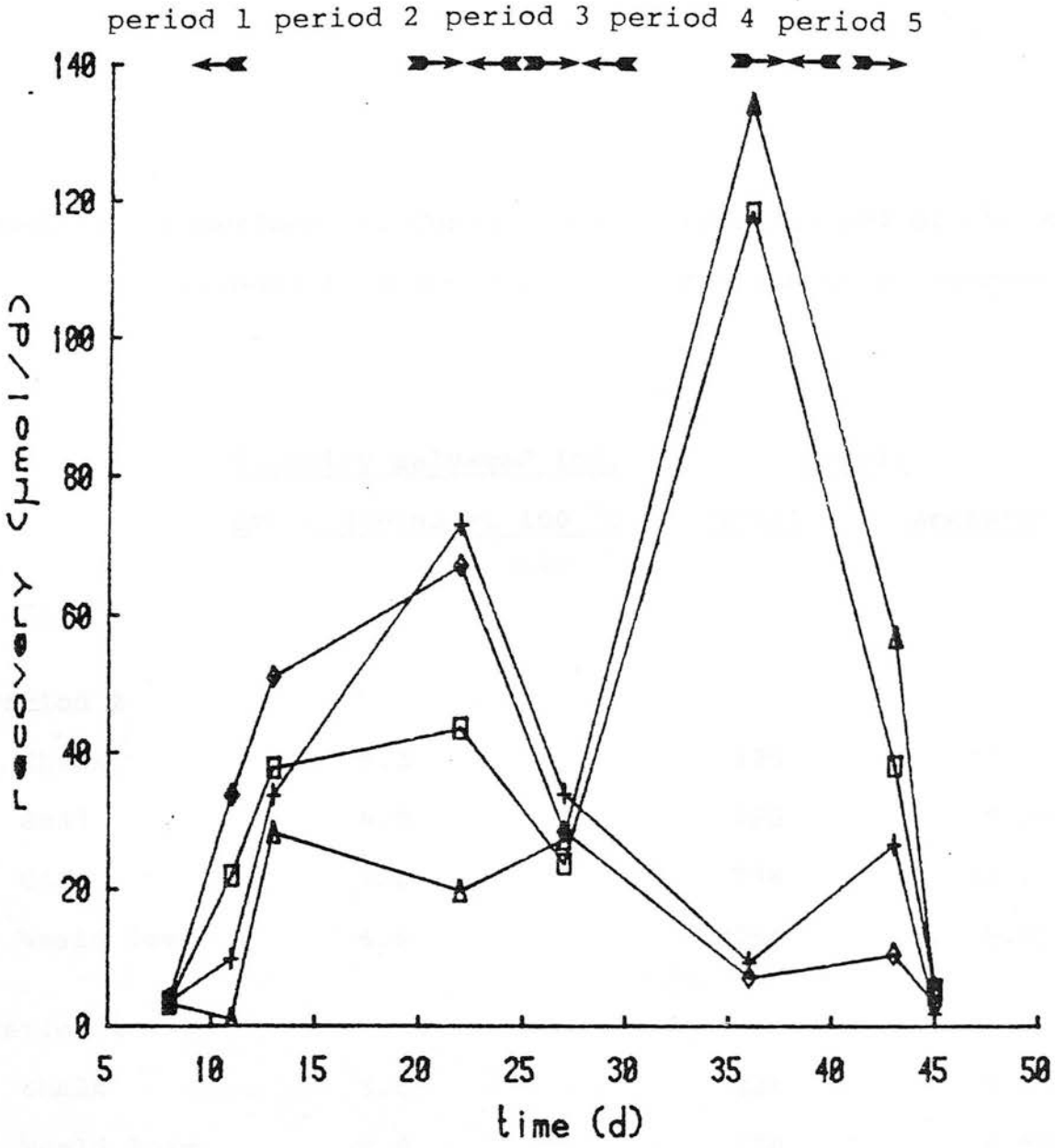


FIG. 6.5 Experiment 4. Recoveries of iron (μmol/d) for vessels 1-4 given 7.22 g DM low cobalt hay(2)/d during all periods of Experiment 4. Soil supplements given during periods 2 (0.7 g/d) and 4 (1.4 g/d) were as follows:

	Period 2	Period 4
vessel 1 (+)	chalk	---
vessel 2 (△)	sand	chalk
vessel 3 (□)	clay	weald loam
vessel 4 (◇)	weald loam	--- .

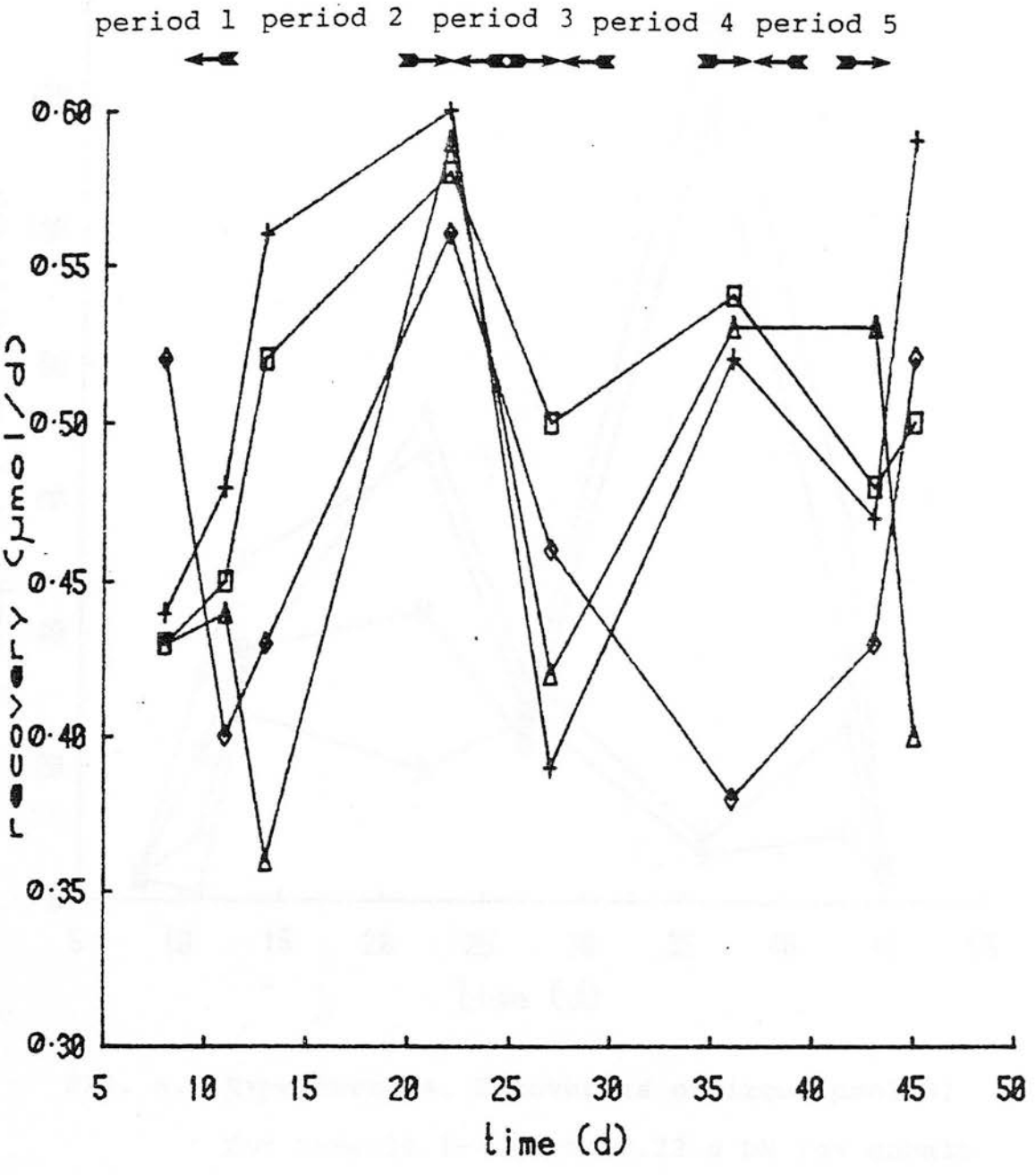


FIG. 6.6 Experiment 4. Recoveries of copper (µmol/d) for vessels 1-4 given 7.22 g DM low cobalt hay(2)/d during all periods of Experiment 4. Soil supplements were given during periods 2 and 4, see FIG. 6.5 for details.

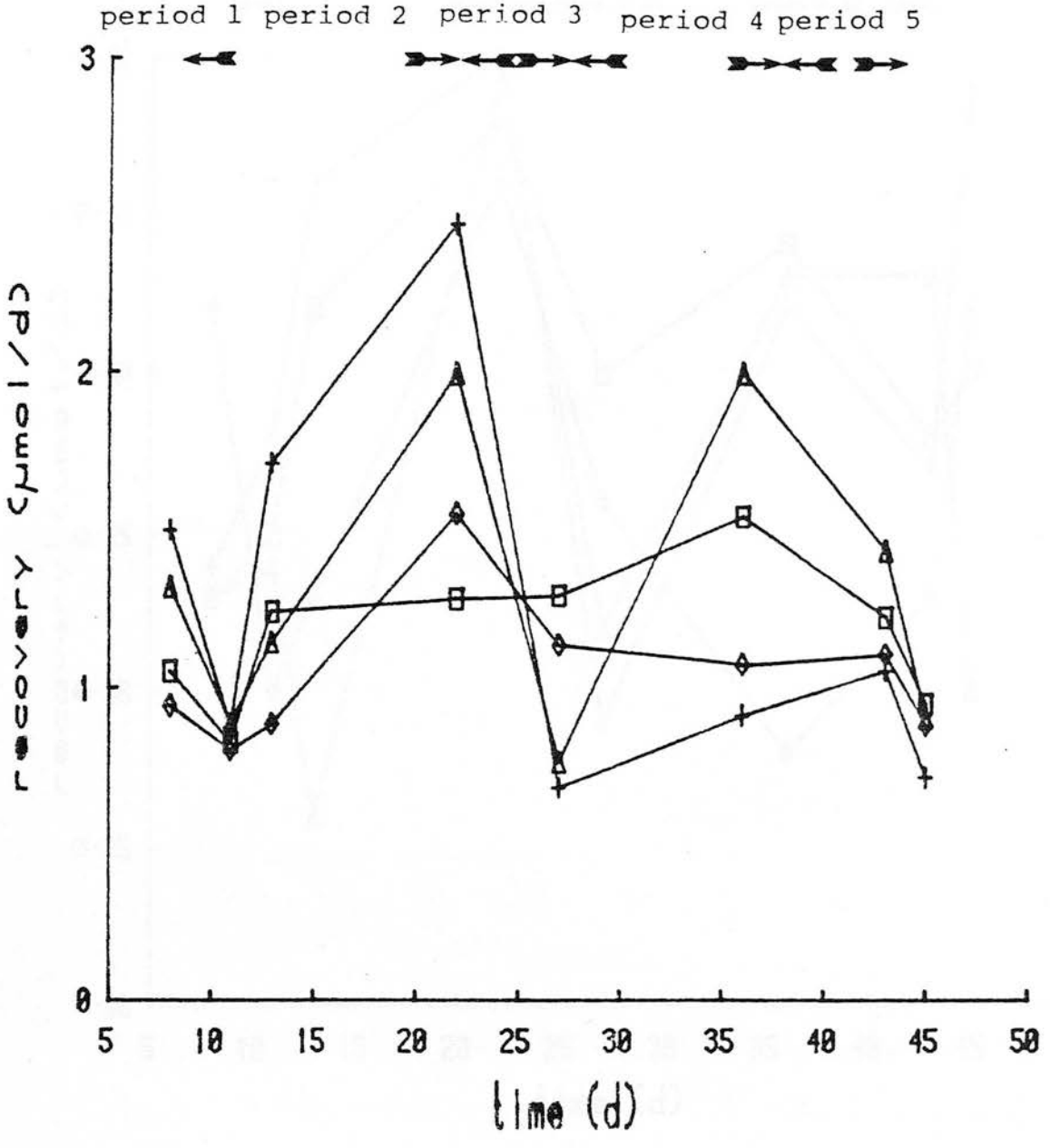


FIG. 6.7 Experiment 4. Recoveries of zinc (µmol/d) for vessels 1-4 given 7.22 g DM low cobalt hay(2)/d during all periods of Experiment 4. Soil supplements were given during periods 2 and 4, see FIG. 6.5 for details.

TABLE 6.8 Experiment 4. Recoveries in effluent (% of daily input from soil) of iron, copper and zinc supplied in soils used in period 2 (0.7 g DM/d) and period 4 (1.4 g DM/d) and measured on the final day of the period.

		Soil			
	Period	chalk	sand	clay	weald loam
Iron	2	30.4	93.9	22.0	21.5
	4	28.6	-	-	19.4
Copper	2	103	392	223	17.5
	4	32.3	-	-	6.01
Zinc	2	153	7980	121	35.7
	4	53.9	-	-	17.0

Cobalt in the salvaged soil residues

Of the 9.8 g of soil added to each vessel during period 2, 54-71 % was recovered on d 22. Values for the total Co content of the soil residues were greater than the initial values for the 2 low Co soils, but less for the 2 high Co soils, while the acetate-extractable Co contents were greater for all but the weald loam (Tables 6.2 and 6.7). At the higher level of supplementation during period 4, recoveries of the chalk and the weald loam were lower than those in period 2 (39.3 vs. 54.1 % and 57.1 vs. 61.2 % respectively) and both measures of soil Co had altered as in period 2 (Table 6.7).

Recoveries of iron, copper and zinc from the effluents during soil supplementation (periods 2 and 4)

During period 2 the daily recoveries of iron (Fig. 6.5), copper (Fig. 6.6) and zinc (Fig. 6.7) increased with all the soil supplements. For none of the elements were the ranking of recoveries on d 22 in accord with the elemental content of the soils and on this day equilibrium appeared to have only been achieved for iron in the sand and clay (Fig. 6.5) and for zinc in the clay (Fig. 6.7). This attainment of equilibrium, in the presence of accumulating soil residues, indicated a rapid release of the element from the soil. All 3 elements were most labile in the sand and least labile in the weald loam, as judged from the proportion of the element recovered in the effluent (Table 6.8).

TABLE 6.9 Experiment 4. Responses (mean value of period minus mean value from corresponding vessel during period 1) of VFA molar proportions (%) and the acetate: propionate ratio, from the cultures in vessels 1-4 when they were supplemented with soil at 0.7 (period 2) or 1.4 (period 4) g/d. Mean values were derived from 3 measurements and are detailed in APP. 4.2.

	Soil	Level of supplement (g/d)			
		0.7		1.4	
		Vessel		Vessel	
Acetic acid					
	chalk	1)	-15.2	2)	11.2
	sand	2)	6.90		-
	clay	3)	9.90		-
	weald loam	4)	11.0	3)	11.4
Propionic acid					
	chalk	1)	6.30	2)	-7.00
	sand	2)	-7.50		-
	clay	3)	-7.40		-
	weald loam	4)	-8.40	3)	-6.40
Total butyric acid					
	chalk	1)	6.30	2)	-3.20
	sand	2)	0.100		-
	clay	3)	-2.10		-
	weald loam	4)	-1.10	3)	-2.60
Total valeric acid					
	chalk	1)	2.40	2)	-1.23
	sand	2)	-0.90		-
	clay	3)	-0.40		-
	weald loam	4)	1.31	4)	-2.13
Acetate: propionate					
	chalk	1)	-1.20	2)	1.15
	sand	2)	1.06		-
	clay	3)	1.16		-
	weald loam	4)	1.59	4)	1.09

In period 4, copper recovery from the chalk (Fig. 6.6) and zinc recovery from the weald loam (Fig. 6.7) may have achieved equilibrium. Only iron recoveries increased in proportion with the increased soil input and for the other elements the percentage recoveries decreased substantially (Table 6.8).

Effects of soil supplements upon VFA patterns (periods 2 and 4)

The addition of 0.7 g/d chalk to vessel 1 appeared to produce responses that were different from those of the other soils (Table 6.9), decreasing acetate and increasing propionate proportions and so reducing the acetate: propionate ratio by 43 % (Table 6.9). These were associated with a depressed total VFA and acetate production (App. 4.3). However, adding either the chalk or the weald loam (vessels 2 and 3 respectively) at 1.4 g/d increased the acetate and decreased the propionate proportions (Table 6.9). This suggested that the results of period 2 were confounded by vessel differences, acetate production being considerably higher in vessel 1 than in the other vessels before soil was added (Table 6.4). The common response was for soil supplementation to be accompanied by increases in the acetate and decreases in the propionate proportions, i.e. a move towards restoration of the "normal" fermentation of roughage. The pH of vessel fluid in compartment 1 (ca. pH 7.20) was not affected by the addition of soils.

TABLE 6.10 Experiment 4. Analogue production (pmol/d) during periods 1, 2, 4 and 5. See TABLE 6.1 for details of treatments.

		Vessel			
		1	2	3	4
Day	Period				
4		6790	3180	3630	3900
6	1	2130	1950	351	66.0
8		1320	59.0	383	484
18		2270	447	5880	775
21	2	8680	1490	5380	5460
22		7490	2350	10800	5010
31		2200	3090	3750	916
36	4	1410	2730	3380	674
37		1690	3100	2460	786
40		2100	3060	2990	2160
42	5	4410	3360	3230	2540
43		2570	3380	3660	2770

Vitamin B12 synthesis when soil was withdrawn (periods 3, 4 and 5)

During period 3, withdrawal of the chalk and weald loam from vessels 1 and 4 was followed by a rapid decrease in Cbl production to levels similar to those at the end of period 1 (Figs. 6.2, 6.3, App. 4.1) and the Cbl content of the washings was also reduced (Fig. 6.4). After the weald loam treatment (0.7 g/d) during period 2 both Cbl production and the Cbl content of the washings achieved minimum values by d 27, but after the chalk supplement (0.7 g/d) the minimum value for the washings was achieved 11 d later than production, on d 37 (Fig. 6.4) and was associated with wide variation in Cbl production throughout the remainder of the experiment.

The initial decrease in analogue production from the culture in vessel 4 (84.3 %, d 37 vs. d 22, Table 6.10) after the withdrawal of the weald loam was not sustained and production was 5.7 times the resting value by the end of period 5 (d 8 vs. d 43, Table 6.10). Similarly, analogue production from the culture in vessel 1, which had previously been given chalk, varied greatly (Table 6.10) and on d 43 the value was 94.7 % greater than the resting value on d 8.

Withdrawal of the sand and clay supplements from vessels 2 and 3 during period 3 (d 23-27) reduced Cbl production by 24 and 23 % respectively (App. 4.1), to levels slightly in excess of the resting values; however, over the same period the Cbl content of the washings increased by 42 and 48 % respectively (Fig. 6.4). After the second period of soil

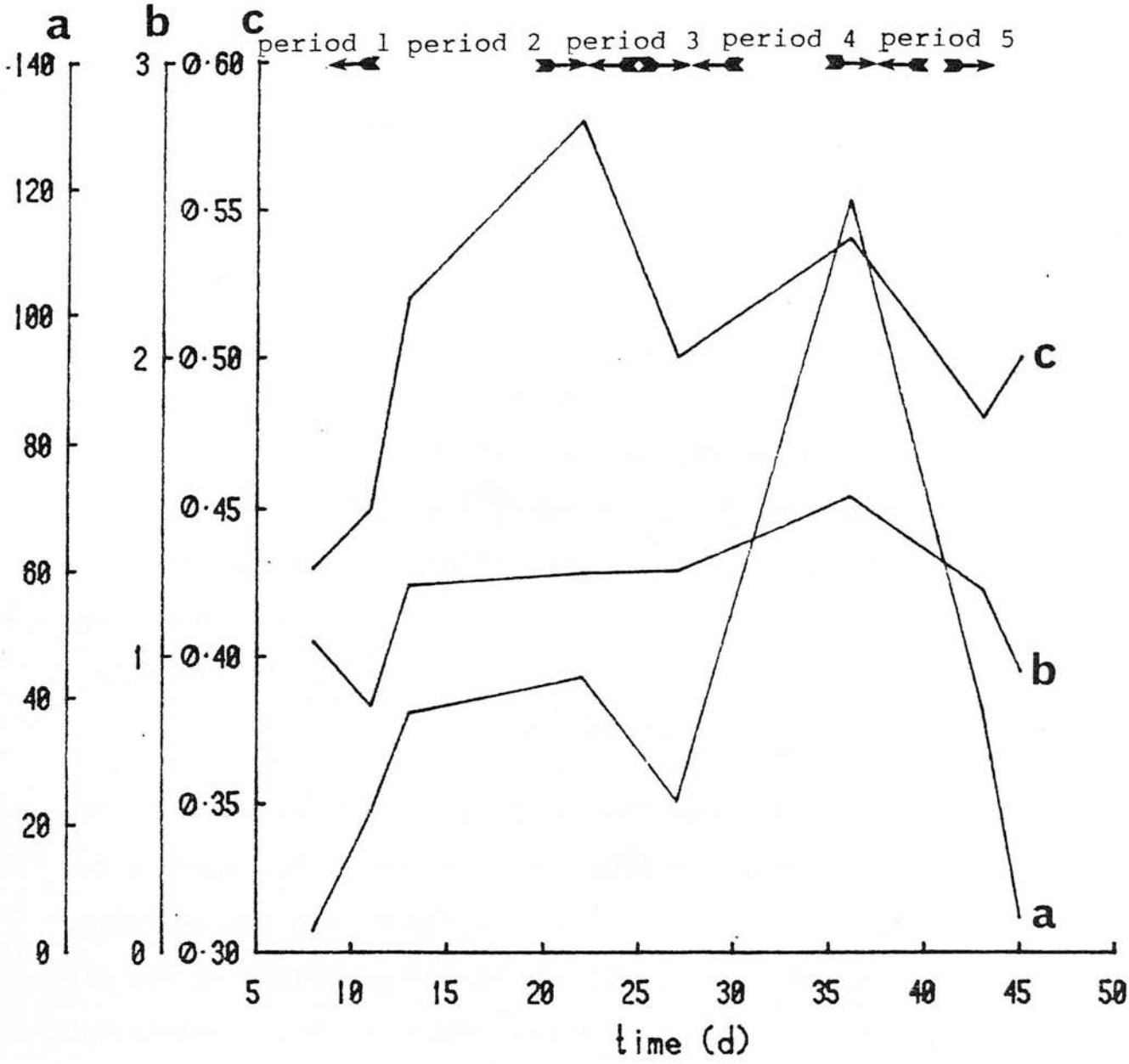


FIG. 6.8 Experiment 4. Recoveries (µmol/d) of iron (a), copper (c) and zinc (b) for vessel 3 given 7.22 g DM low cobalt hay(2)/d. Soil supplements were given during periods 2 (0.7 g clay/d) and 4 (1.4 g weald loam/d).

supplementation, withdrawal of the soils (period 5) was associated with a drop of 40 % in Cbl production within 3 d, but after 6 d values were still approximately 5 times the resting values of period 1 (App. 4.1). The Cbl contents of the washings did not decrease consistently and on d 43 were 15-20 times the resting values of d 8 (Fig. 6.4). Analogue production in either vessel did not decline (Table 6.10).

Iron, copper and zinc recoveries when soil was withdrawn (periods 3, 4 and 5)

Iron (Fig. 6.5), copper (Fig. 6.6) and zinc (Fig. 6.7) recoveries fell when the soil supplements were omitted. For iron, these decreases were rapid for all soils used and levels close to resting values were achieved within 8 d. Similarly, zinc recoveries stabilised quickly. The increases in copper recoveries from the effluent during soil supplementation were much smaller than those for iron and zinc and therefore the effects of withdrawal were hard to establish. The relative recoveries of iron, copper and zinc during 2 phases of soil withdrawal (vessel 3) are shown in Fig. 6.8.

Fermentation when soil was withdrawn (periods 3, 4 and 5)

With the cessation of soil supplementation during period 3, both VFA proportions and production were generally similar to the values of period 1 (Apps. 4.2, 4.3), e.g. the acetate: propionate ratio increased after withdrawing chalk from vessel 1 but decreased after withdrawing the other soils. A process of equilibration continued throughout periods 4 and 5 and by the end of the experiment

TABLE 6.11 Experiment 4. Individual VFA molar proportions (%), acetate: propionate ratio and VFA production (mmol/d) for the cultures in vessels 1-4, given low cobalt hay(2) at 7.22 g DM/d during period 1 (TABLE 6.4) and compared with mean values during period 5 (d 40, 42 and 43).

	Vessel				
	1	2	3	4	Mean ± s.d. (n=4) period 5
Proportions					
Acetic acid	58.4	49.0	48.3	50.4	59.1 ± 1.31
Propionic acid	21.3	27.4	27.1	25.6	19.1 ± 1.43
Total butyric acid	12.9	14.0	13.5	13.9	12.3 ± 1.35
Total valeric acid	7.24	9.64	10.7	9.93	9.62 ± 2.04
Acetate: propionate	2.77	1.80	1.79	1.98	3.11 ± 0.207

TABLE 6.12 Experiment 4. Effects of vessel differences and soil supplementation on mean ADMD and AOMD values (n=3) for 7.22 g DM low cobalt hay(2)/d given to vessels 1-4. Values for periods when no soil supplements were employed are underlined. (see TABLE 6.1 for details of treatments)

		Vessel			
		1	2	3	4
	Period				
ADMD					
	1	0.586	0.571	0.567	0.575
	2	0.549	0.558	0.569	0.578
	3	0.591	0.526	0.586	0.566
	4	0.602	0.546	0.561	0.538
	5	0.606	0.598	0.584	0.601
AOMD					
	1	0.594	0.581	0.577	0.586
	2	0.573	0.582	0.586	0.604
	3	0.603	0.533	0.597	0.578
	4	0.612	0.582	0.595	0.549
	5	0.615	0.617	0.598	0.610

the "atypical" fermentations exhibited by the cultures in vessels 2, 3 and 4 at the start of the experiment (Table 6.4) were no longer evident (Table 6.11), e.g. large increases in the acetate: propionate ratios in these cultures had occurred, which had been caused by increases in the acetate and decreases in the propionate molar proportions.

Digestibility of hay ration when unsupplemented and soil supplemented

During period 1 both ADMD and AOMD were higher in vessel 1 than in vessels 2-4. This difference may have persisted throughout the experiment because during the subsequent periods (3, 4 and 5) when vessels 1 and 4 were unsupplemented the digestibility of the substrate in vessel 1 was always greater than that in the other vessels (Table 6.12). There was no indication that soil supplementation had a consistent effect upon digestibility (Table 6.12), the values for which were in general agreement with the *in vitro* value determined at the East of Scotland College of Agriculture (0.568, Table 3.1).

Discussion

Resting values for Cbl and analogue production, VFA synthesis and digestibility (periods 1 and 5)

The differences in Cbl production during period 1 between the culture in vessel 1 and those in the other 3 vessels persisted throughout the experiment, the levels of production being 2.3-8.6 times greater for vessel 1 on d 43 than for the other vessels. However, the differences in analogue production, VFA synthesis and digestibility between cultures were less persistent. Consideration of all the parameters measured in periods 1 and 5 suggested that the fermentation in vessel 1 was not abnormal and that the cultures of vessels 2-4 eventually became more representative of a roughage fermentation.

The equilibration of the cultures may have been hastened by mixing the fluid from compartment 1 of vessels 1, 3 and 4 on d 25 and so causing a partial redistribution of the predominant microbes. However, the c.v. for the acetate: propionate ratio and Cbl production from all 4 vessels on d 8 and d 27 were 21.9 vs. 17.5 % and 84.5 vs. 82.1 % respectively, suggesting that any such effect was slight.

Values for the Cbl content of the washings and their correlation with production on d 8 illustrated the importance of variation in this compartment between vessels and variation in production. The importance of both compartment 2 and individual variation between cultures was indicated by the significant ($p<0.01$) negative correlation between the Cbl content of the washings and the acetate: propionate ratio for the culture in vessel 1, when data

from all periods of the experiment were considered, whereas consideration of similar data from vessels 2, 3 and 4 showed a significant ($p<0.05$) positive correlation. Because of the role of vitamin B12 in acetate, and possibly propionate, synthesis a direct link between Cbl production and VFA production may exist. However, in this experiment the responses in vitamin B12 synthesis to the supplementation with soil may have been confounded by culture differences.

Recoveries of iron, copper and zinc during period 1

The decrease in values for all 3 elements (Fig. 6.1) during period 1 was due to the low trace element content of the low Co hay(2) when compared to the innoculum. Resting values for iron, copper and zinc achieved by d 8, were 21.2, 68.0 and 113 % of the daily input of these elements in the hay and the value for zinc fell to 80.0 % by d 11. These results may indicate different degrees of liberation of these elements from the herbage during digestion and Suttle et al. (1982) found that zinc, the most labile element of the 3, was well absorbed by sheep. Differential retention of the elements within the vessel by microbial accumulation, in compartment 2 particularly, and subsequent recycling of the elements cannot however be ruled out. All 3 elements have long been known to be accumulated by rumen microbes (Mitchell and Tosic, 1949).

Effects of soil supplementation upon vitamin B12 synthesis (periods 2 and 4)

Chalk The addition of this soil, which was adequate with regard to total but not acetate-extractable Co, increased Cbl production by 364 % at 10 % DMI and by 498 % at 20 % DMI, and caused even greater increases in analogue production. Equivalent increases in the Co concentration of the hay(2) (832 nmol Co/kg DM) would ensure that the Co supply was well above the ARC (1980) minimum requirement for Co (1,870 nmol/kg DM), i.e. a $\frac{1870}{832}$ increase (= 225 %) would satisfy ARC requirements. These responses in vitamin B12 synthesis may have resulted from the increased Co supply or from an indirect effect on cultures. However, chalk, like the other soils, did not alter the pH of compartment 1 at either level of supplementation and the magnitude of the effect makes a direct Co effect more likely.

The "rumen availability" of ingested soil Co from chalk during period 2 (3.44 % was incorporated into Cbl and analogues) was greater than "plant availability" (i.e. acetate extractable Co was 0.406 % of the total soil Co); this may reflect vastly different chemical environments influencing extraction. The Co supply to plants might also be restricted by the high levels of manganese (Adams et al., 1969) and OM (COSAC, 1982) in the soils, as listed in Table 6.1. In Rusitec, the strict reducing conditions of the culture may have attenuated the binding capacity of manganese (as the oxide) with which Co associates and soil OM would have been subject to microbial digestion. The

proportion of the soil Co incorporated into Cbl or analogues from the chalk was nevertheless small compared with that of Co from the unsupplemented hay in vessel 1 (31.3 %, Table 6.3).

The increases in Cbl and analogue production (Table 6.5) and the Cbl content of the washings (Fig. 6.4) for the 1.4 g/d chalk supplement were surprisingly less than those of the smaller supplement (0.7 g/d) in period 2 and only 0.712 % of the Co was used for vitamin B12 synthesis. However, the differences between the cultures in vessels 1 and 2 during period 1 have already been mentioned and the smaller response in vitamin B12 synthesis during period 4, when conversion efficiencies were considered, may reflect a difference between cultures, the culture in vessel 1 being a more prolific synthesiser of the vitamin than the other cultures. At both levels of supplementation, the ratio of responses in analogue: Cbl production was greater than the ratio produced by the unsupplemented hay in the same vessel (3.37 and 3.95 vs. 2.39).

Sand This soil was deficient when considered in terms of either total or acetate-extractable Co and this was not unexpected as Co-deficiency occurs on such soils in many regions of the world (Latteur, 1962). However, 24.0 % of the added Co was incorporated into vitamin B12 and supplementation at 10 % DMI produced a 96.0 % increase in Cbl production, which was not quite sufficient to achieve an ARC (1980) standard of adequacy, as calculated earlier. Furthermore, the response was attained in a culture which produced low levels of Cbl during period 1. The greater

response in analogue production to supplementation (analogue: Cbl ratio of 13.1) may have been due to an interaction between the soil and the culture, particularly as the other culture with low resting Cbl values (vessel 3) also showed an enhanced response in analogue production with the introduction of soil. Both cultures may have responded to the prior Co depletion by a change in microbial composition, e.g. an increase in species producing a preponderance of analogues.

Clay The responses in Cbl production (Fig. 6.2) and the Cbl content of the washings (Fig. 6.4) to the inclusion of this soil at 10 % DMI were quantitatively similar to that of the sand supplement. The clay had far higher levels of both total and acetate-extractable Co (Table 6.2), but the responses in Cbl production and the Cbl content of the washings were proportionally less, only 0.923 % of total Co from the clay being incorporated into vitamin B12. The clay, like the sand, was administered to a culture with a low capacity for Cbl and analogue synthesis. Clays have a high cation exchange capacity and can adsorb Co as well as incorporate it more permanently within the soil lattice (West, 1981) and this factor may have influenced the liberation of Co within the culture.

As with the sand, there was a greater response in analogue than Cbl production during period 2 and this preferential synthesis of analogues from sequestered soil Co creates difficulties in the assessment of the availability of soil to ruminants since there might subsequently be microbial recycling of analogues to Cbl, possibly an important factor in the absorption of the vitamin.

Weald loam The 2 vessels in which weald loam was used had similiar resting Cbl production levels and Cbl production doubled with the doubling of the soil supplement, increases of 335 and 670 % occurring at levels of 10 and 20 % DMI respectively. The efficiency of Co incorporation into vitamin B12 was high but declined with increased input from 26.5 % to 8.84 % in periods 2 and 4 respectively. The effective Co input was well in excess of the ARC requirements, even at the lower inclusion rates. Increases in analogue production were greater than those in Cbl, particularly at the lower inclusion rate (984 and 736 % in periods 2 and 4 respectively), but the relative responses were more in accord with the chalk than the other soils.

That the weald loam was a far better stimulator of Cbl synthesis during period 2 than the clay (conversion efficiencies 2.90 vs. 0.016 %, Table 6.6) was also suggested by the greater increase of Cbl in the washings when the weald loam was added to the same culture (128 vs. 20.0 pmol/d), albeit at a higher level. The low level of Cbl production in the presence of clay during period 2 suggested that any carryover of sequestered Co would have been small. The high levels of OM and iron in the weald loam, relative to the other soils (Table 6.2), did not apparently restrict the availability of Co.

Using the responses in Cbl plus analogues as an indicator (i.e. the combined conversion efficiencies), the weald loam would appear to have been the most labile and the clay the least labile source of Co (Table 6.6). However, these deductions contrast with the values for the total Co in the "salvaged soil" (Table 6.7), from which the weald loam appeared to have accreted Co and the clay to have released Co.

The varying degrees of efficiency with which soil Co was utilised for vitamin B12 synthesis (0.016-2.90 % for Cbl and 0.568-23.6 % for analogue production) encompassed the values determined for the Co nitrate supplement in Experiment 2b (1.29 and 15.7 % for Cbl and analogue respectively) and both the sand and the weald loam, when supplemented with soil at 0.7 g DM/d, had higher efficiencies. The sand and the weald loam were not exceptional with regard to either total and acetate-extractable Co measurements and the latter are therefore unreliable estimates of the amount of Co that is available for utilisation by microbes in rumen cultures.

Cobalt contents of the "salvaged" soils The results for the salvaged material appear to suggest that, despite acting as a source of Co to the microbes, the soils concentrated the element. While only the sand and the weald loam increased their total Co content, the chalk, the sand and the clay increased their acetate-extractable levels. These inconsistencies may have been the result of:

1. chemical transformation of the Co associated with the soil, produced by the reducing conditions (see Fig. 1.2), affecting both the total and the acetate-extractable measurement.
2. an uneven distribution of Co within a soil, e.g. more Co associated with the larger or denser particles that were recovered.
3. contamination, e.g. the abrasive action of the soil on the steel rods.
4. removal of the soil OM by microbial digestion.

Recoveries of iron, copper and zinc from soil supplements (periods 2 and 4)

The recoveries of the elements were influenced by the soil; those from the weald loam were always lowest and those from sand were the highest (Table 6.8). The final day recoveries were often greater than 100 % because soil was accumulating in the cultures. There were also differences between the elements; iron recoveries were generally lower than those for the other 2 elements. Such differences probably resulted from inadequate liberation from the soil and the considerable quantity of soil salvaged supported this possibility. These differences, as well as those in Co availability for vitamin B12 synthesis, could be related to the physical and chemical properties of the soil, e.g. the association of Co with manganese, microbial sequestration, particle size and density.

Effects of soil withdrawal upon vitamin B12 synthesis (periods 3, 4 and 5)

Only in vessel 4 was withdrawal of the soil associated with decreases in both Cbl production and the Cbl contents of the washings to the initial resting values and even then the analogue production remained high during period 5. The maintenance of high levels of Cbl production and Cbl in the washings from period 3 onwards for vessel 1 highlighted the different behaviour of this culture. During period 5 the cultures in vessels 2 and 3 produced levels of Cbl and analogue production far greater than those of period 1. That the 6 d of period 5 was insufficient to produce an equilibrium value became apparent in the following experiment (Chapter 7).

Support for an extended carryover effect of the soil involving the microbes comes from the observation that the Cbl content of the washings (Fig. 6.4) and analogue production (App. 4.1) were maintained at levels far above the resting values after withdrawal of the soil, whereas Cbl production tended to decline (Fig. 6.3). This was possibly due to the effects of stored Co in the microbially-rich compartment 2 and the subsequent synthesis of Cbl and analogues. These factors could also explain the lack of response in production for the first 5 d after supplementation commenced (Fig. 6.2).

Conclusions

1. The introduction of soil supplements was associated with increases in the acetate and decreases in the propionate proportions in 3 of the 4 cultures; these cultures had started the experiment with atypically low acetate: propionate ratios.
2. There may have been an underlying trend throughout the experiment for the atypical cultures to align with the fourth culture in producing VFA patterns representative of a roughage fermentation.
3. Despite the complications of culture differences, the 4 soils (a chalk, a sand, a clay and a weald loam) probably differed in their capacity to supply Co for vitamin B12 synthesis in the order weald loam> sand> chalk> clay, which was unrelated to either the total or acetate-extractable content of the soils.

4. For all 4 soils the Co was less efficiently incorporated into Cbl than that from a low Co hay(2) substrate given to the same cultures.

5. Increases in analogue production were far greater than those in Cbl, particularly for the sand and the clay, so complicating an assessment of biological availability.

6. Iron recoveries were generally lower than those of copper and zinc and all 3 elements were least labile in the weald loam and most labile from the sand.

CHAPTER 7

EXPERIMENT 5a. THE INFLUENCE OF A PROPIONATE ENHANCER UPON RUMINAL VITAMIN B12 SYNTHESIS

Introduction

In previous experiments there was evidence of an association between Cbl production and the fermentation characteristics of the cultures. Firstly, supplementary Co was incorporated less efficiently into Cbl when barley, rather than hay, was used as a substrate. Secondly, in cultures given barley, which produces a "propionate" fermentation, Cbl production was negatively correlated with the proportion of propionic acid in the total VFA.

Monensin, an acidic compound produced by Streptomyces cinnamonensis, enhances the molar proportion of propionate, with a concomitant decrease in methanogenosis, in the rumen fermentation of roughage- and concentrate-based diets, both in vitro and in vivo (Chalupa, 1980): it is used extensively for increasing the efficiency of feed conversion (Wilkinson et al., 1980). The action of monensin is considered to be mediated through its toxicity to certain microbial species (Chen and Wolin, 1979). As vitamin B12 is required for propionate metabolism, acetate synthesis and methanogenesis (Table 1.4), an experiment was designed in which the effects of monensin on vitamin B12 production in Rusitec were studied, using hay as the substrate.

TABLE 7.1 Experiment 5. Experimental design used in Rusitec to investigate the effect of monensin (334 nmol/d) upon vitamin B12 and VFA production in the presence (47.6 nmol/d) and absence of supplementary cobalt. Also studied was the effect of a cobalt chelate upon the same rumen parameters.

		Vessel			
		1	2	3	4
Period	Days				
Experiment 5a					
trans.	1-6*	<-------no supplement--------->			
1	7-18	<-------- + monensin --------->			
2	19-30	<-------- + monensin --------->			
		<--------- + cobalt ---------->			
3	31-42	<-------- + monensin --------->			
		<- + cobalt --><-- no cobalt-->			
Experiment 5b	43-50	<-------- + monensin --------->			
		<- + Co EDTA-><-- no cobalt-->			

* = see text for details

Experimental procedure

As vessel differences had been apparent in previous experiments it was decided to infuse monensin into all 4 Rusitec cultures and to compare the efficiencies of Cbl and analogue synthesis, in the presence and absence of supplementary Co, with values obtained from earlier experiments.

The experimental design is outlined in Table 7.1. The low Co hay(2) used in Experiment 4 (Table 3.1) was retained as the basal ration and the cultures from that experiment were continued as Experiment 5. Soil supplements had been withdrawn for 6 d (vessels 2 and 3) or 21 d (vessels 1 and 4) prior to supplementation with monensin (the transition period). In period 1 (d 7-18), monensin was infused into all 4 vessels at 334 nmol/d (equivalent to 33 mg sodium monensin/kg DM), via the artificial saliva, after an initial dissolution of the monensin in 50 % methanol. This is a level typically employed *in vivo*, but it should be noted that the dispersal of monensin in the liquid phase of Rusitec would dilute it more than would occur in the rumen (Wallace *et al.*, 1981). In period 2, Co was infused at 47.6 nmol/d (equivalent to 0.40 mg Co/kg DM) with monensin, into all 4 vessels for a further 12 d (d 19-30). The vessels were then paired and the Co infusions into vessels 3 and 4 stopped (period 3; d 31-42).

The importance of compartment 2 in vitamin B12 synthesis had been recognised in previous experiments; therefore, samples of both effluent and washings were collected regularly throughout this experiment, during which the

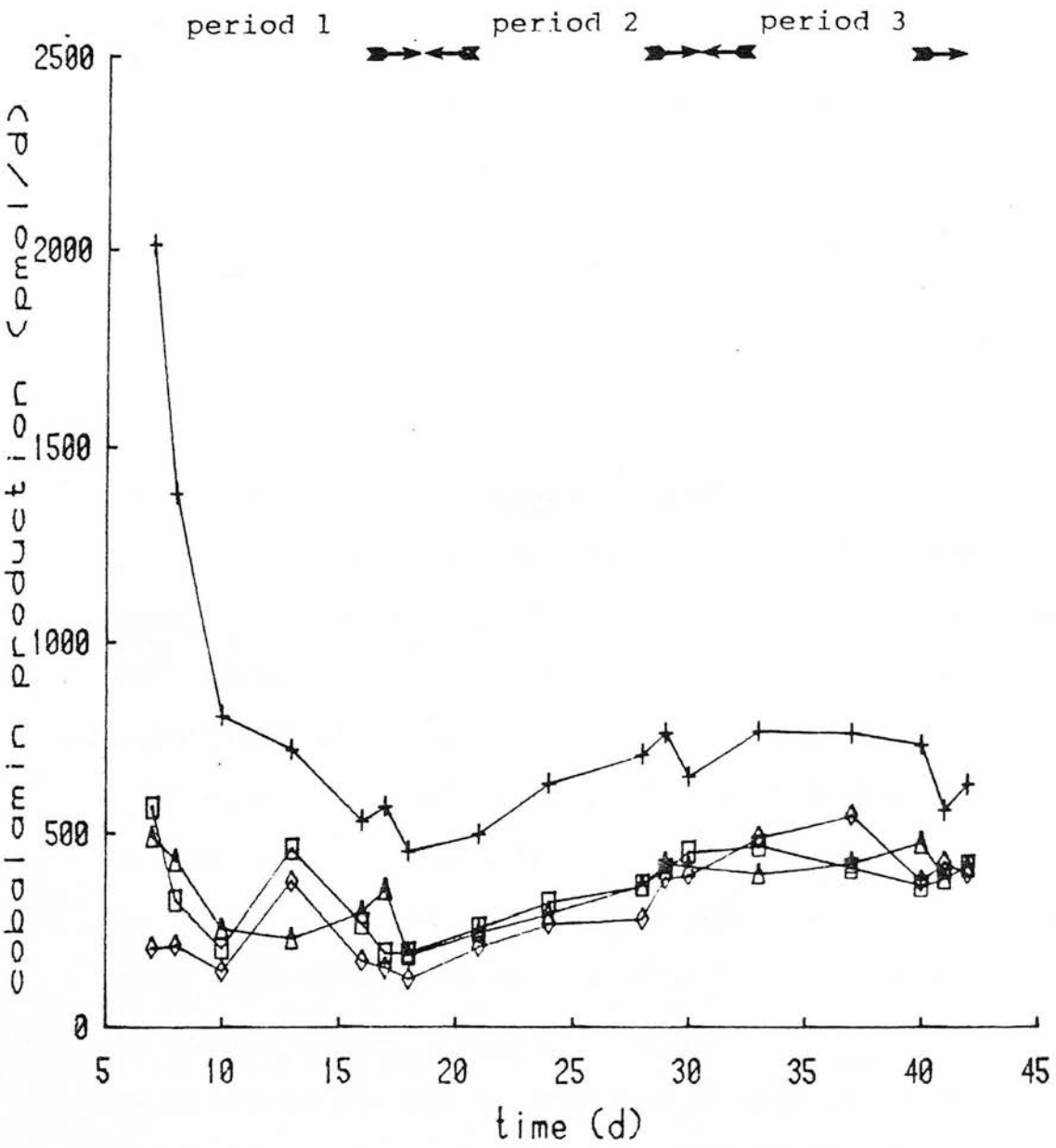


FIG. 7.1 Experiment 5a. Cobalamin production (pmol/d) for vessels 1-4 given 7.22 g DM low cobalt hay(2) and 334 nmol monensin /d. A cobalt supplement of 47.6 nmol Co/d was given to all vessels in period 2 and to vessels 1 and 2 only in period 3. (vessels: 1 = +, 2 = △, 3 = □, 4 = ◇)

TABLE 7.2 Experiment 5a. Analogue production (pmol/d) during the transition period (no supplement) and during 3 periods of monensin infusion (334 nmol/d) for vessels given 7.22 g DM low cobalt hay/d. Cobalt was infused into all vessels during period 2 and into vessels 1 and 2 only during period 3.

		Vessel			
		1	2	3	4
Period	Day				
	3	2090	3060	2990	2160
transition	5	4410	3360	3230	2340
	6	2570	3380	3660	2770
	16	1950	1870	1640	1810
1 (no Co)	17	718	1030	654	358
	18	774	875	1350	472
	28	1930	1940	2110	1560
2 (+ Co)	29	2300	2200	1910	1040
	30	1710	1460	1780	1270
	40	6560	6490	2880	6410
3 (± Co)	41	6810	7100	5730	4310
	42	8950	4600	3740	1310

TABLE 7.3 Experiment 5a. Responses to monensin infusion of cobalamin and analogue production (pmol/d) (where responses are mean value of period 1 minus resting value from Experiment 4), when 4 Rusitec cultures were each given 7.22 g DM low cobalt hay/d plus a monensin supplement (334 nmol/d) throughout. A cobalt supplement was also used in periods 2 and 3. The values used are means of values from samples taken on the following occasions: period 1 = d 16, 17 and 18, 2 = d 28, 29 and 30, 3 = d 40, 41 and 42.

		Vessel			
		1	2	3	4
Responses	Period				
Cobalamin	1 (no Co)	-32.0	131	103	-28.0
	2 (+ Co)	152	251	294	175
	3 (± Co)	87.0*	138*	275	222
Analogues	1 (no Co)	-574	894	617	-69.0
	2 (+ Co)	660	1810	1550	806
	3 (± Co)	6120*	6000*	3740	3530

*= + cobalt

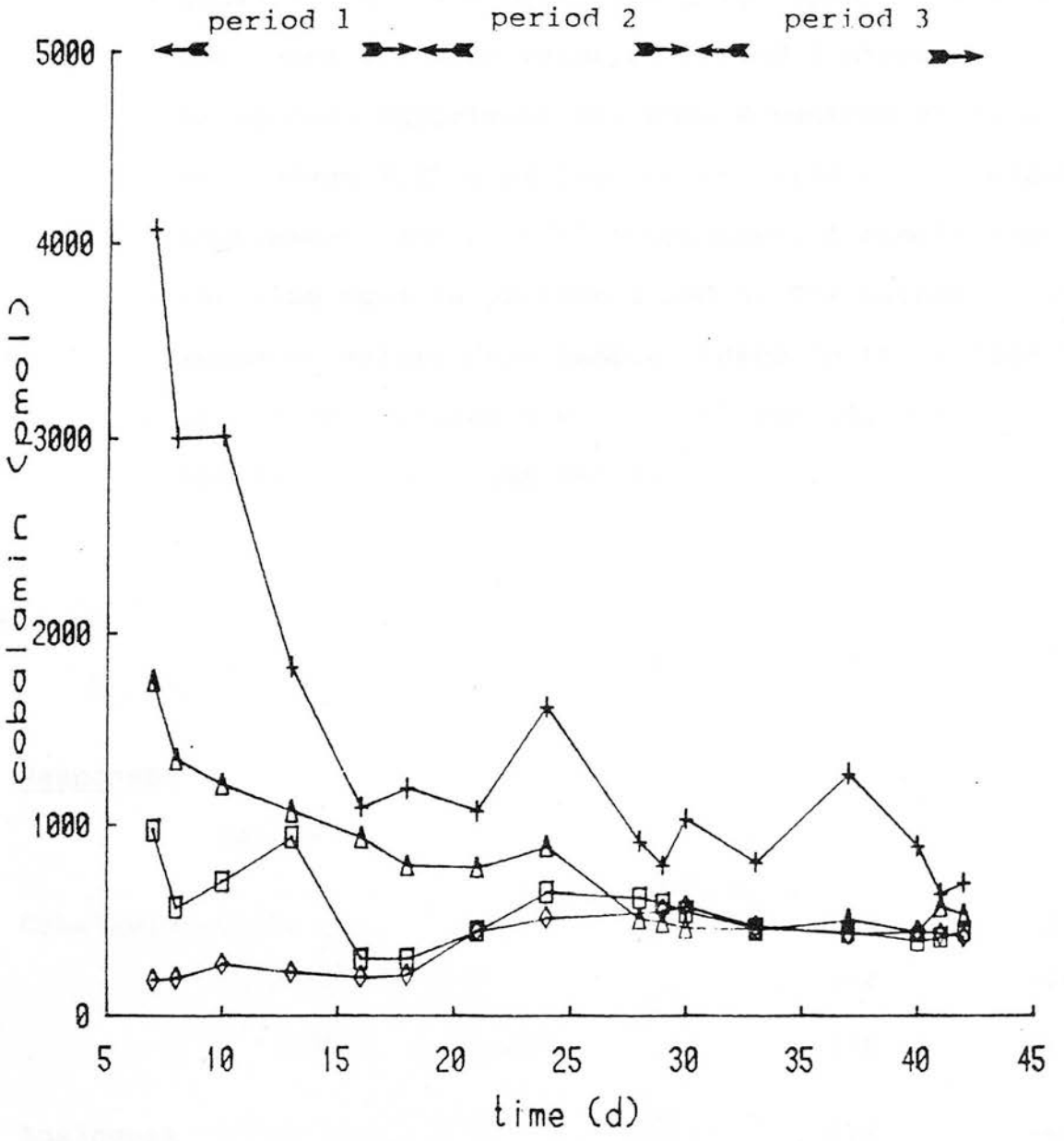


FIG. 7.2 Experiment 5a. Cobalamin content (pmol) of the washings from vessels 1-4 given 7.22 g DM low cobalt hay(2) and 334 nmol monensin /d.
A cobalt supplement of 47.6 nmol Co/d was given to all vessels in period 2 and to vessels 1 and 2 only in period 3. (vessels: 1 = +, 2 = △, 3 = □, 4 = ◇)

TABLE 7.4 Experiment 5a. Mean equilibrium values for the cobalamin content of the washings from vessels 1-4 given 7.22 g DM low cobalt hay(1)/d compared with resting values obtained from d 8 of Experiment 4. Monensin (334 nmol/d) was infused into all vessels throughout, with or without cobalt (47.6 nmol/d). Values are the means of samples taken on the following occasions: period 1 = d 16 and 18, 2 = d 29 and 30, 3 = d 42 and 44.

		Vessel			
		1	2	3	4
Supplement	Period				
-	resting	990	89.0	81.7	447
Monensin	1	1140	859	293	204
Monensin + Co	2	908	471	562	559
Monensin ± Co	3	772	544	421	416

dilution rate was maintained at 0.75 /d. In other respects the procedures for assessing the effects of the treatments on Cbl and analogue production and on the patterns of fermentation were those described for Experiments 3 and 4.

Results

Vitamin B12 synthesis

Effects of monensin (period 1) It was clear that differences between cultures in Cbl production (Fig. 7.1, App. 5.1), and possibly analogue production (Table 7.2), persisted beyond the transition period; but Cbl production declined rapidly from the initially high levels in vessel 1 at the beginning of this period and Cbl and analogue production were considered to have stabilised in all vessels by d 16 (Fig. 7.1, App. 5.1) and d 17 (Table 7.2) respectively. The responses to monensin were derived as differences between these stable values for period 1 and the resting values for the same unsupplemented hay, used at the beginning of Experiment 4, and are given in Table 7.3. Monensin did not uniformly influence either Cbl or analogue production, although it is interesting to note that production was decreased in the 2 vessels given 21 d to equilibrate prior to the monensin infusion. The mean conversion efficiences for Cbl and analogue synthesis during this period were 4.86 ± 2.71 % and 18.8 ± 2.84 % respectively.

The Cbl content of the washings may not have equilibrated in vessels 2 and 3 by the completion of period 1 (Fig. 7.2), since the values for these washings were considerably greater than the resting values for these cultures in Experiment 4 (Table 7.4).

Effects of monensin plus cobalt supplements (periods 2 and 3) Infusing Co nitrate at 47.6 nmol/d caused Cbl production from all the cultures to increase (Fig. 7.1) and at the end of period 2 both Cbl and analogue production (Table 7.3) had significantly increased ($p<0.01$), when analysed using the paired t-test. Increases in the Cbl content of the washings occurred in vessels 3 and 4 only during this period (Table 7.4); this also suggested that the minimum values of equilibrium may not have been achieved in all the vessels during period 1.

The continuation of Co supplementation in vessels 1 and 2 during period 3 produced no further increase in Cbl production (Fig. 7.1, Table 7.3) or in the Cbl content of the washings (Table 7.4). The difference between values at the end of periods 1 and 2 (Table 7.3) provided data from which the efficiencies of incorporation of the Co supplement into Cbl could be calculated, the mean ± s.d. being 0.367 ± 0.0783 %. Analogue production was further increased during period 3 in vessels 1 and 2 and the best estimate of the efficiency of analogue production from the Co supplement given with monensin was that from vessels 1 and 2 at the end of period 3; the mean increase over period 1 was 5900 ± 1120 pmol, giving a conversion efficiency for the Co into analogues of 12.4 ± 2.40 %.

Withdrawal of the Co supplement from vessels 3 and 4 (period 3) did not cause a decrease in Cbl production (Fig. 7.1, Table 7.3), but there was a significant ($p<0.01$) 25 % drop in the Cbl content of the washings (Fig 7.2, Table 7.4). Analogue production increased substantially in all

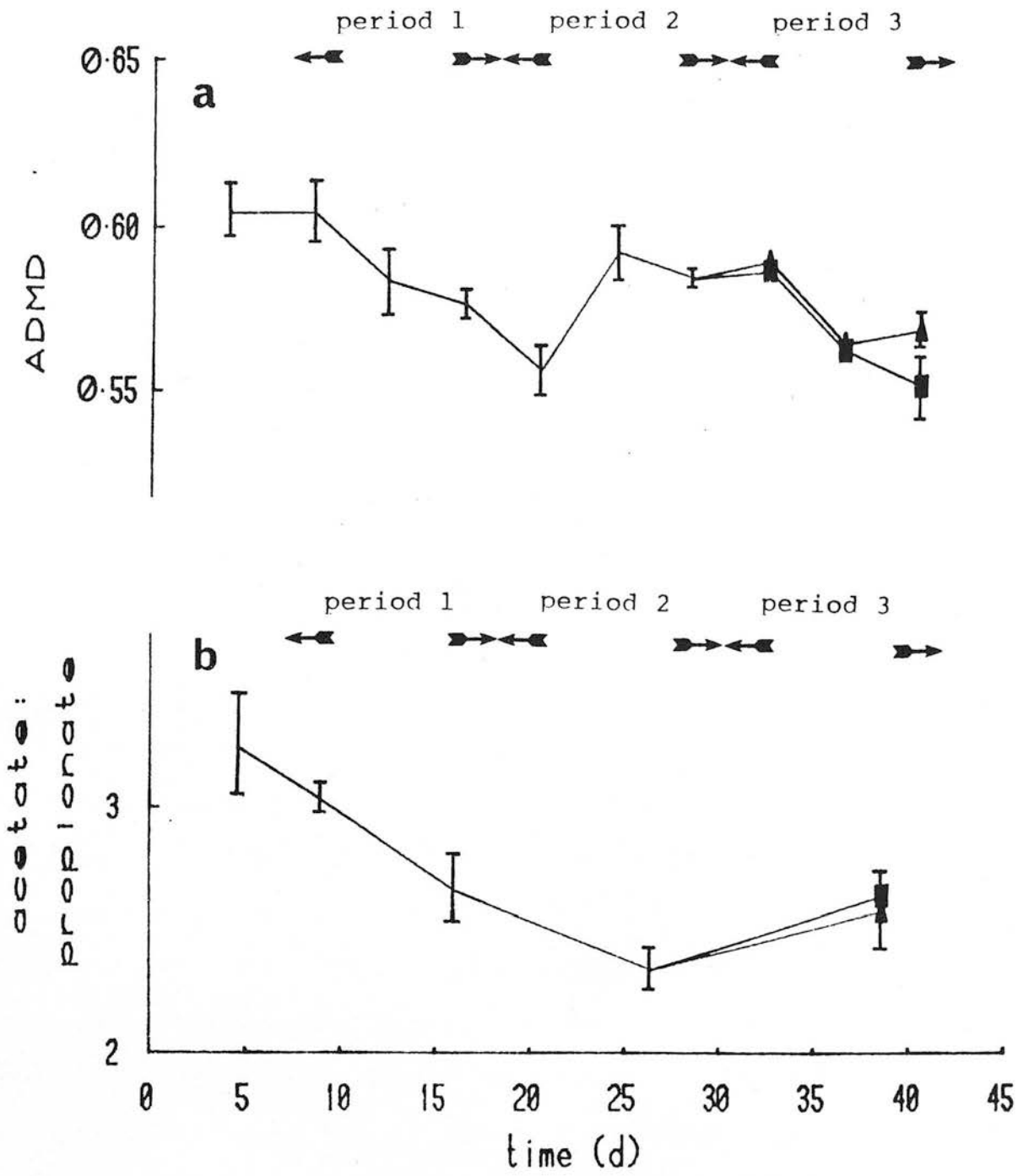


FIG. 7.3 Experiment 5a. Mean (± s.d.) of ADMD (a) and the acetate: propionate ratio (b) for vessels 1-4 in periods 1 and 2; plus mean (± s.d.) of the same measurements for vessels 1 and 2 (▲) and vessels 3 and 4 (■) during period 3. All vessels were given 7.22 g DM low cobalt hay(2) and 334 nmol monensin /d throughout. In addition, a cobalt supplement of 47.6 nmol Co/d was given to all vessels in period 2 and to vessels 1 and 2 only during period 3.

vessels during period 3 (Table 7.3) but the levels attained in vessels 1 and 2 were significantly ($p<0.01$) greater than those in vessels 3 and 4, which no longer received supplementary Co.

It is worthwhile noting that throughout the experiment both Cbl production and the Cbl content of the washings from vessel 1 were higher than values for the other vessels, whatever the treatment (Figs. 7.1, 7.2), whereas vessel differences in analogue production were less obvious.

ADMD of hay

The digestibility of the hay decreased steadily during the first period of monensin supplementation and the difference was significant ($p<0.05$) after 13 d (Fig. 7.3). This decline in values continued for the first 3 d following the introduction of Co, but it was then reversed and values at the end of period 2 were only slightly less than initial values.

In period 3, the Co supplement was withdrawn from vessels 3 and 4, but in all vessels ADMD eventually declined.

VFA synthesis

The transition period between Experiments 4 and 5 provided baseline values for VFA synthesis against which the periods of monensin supplementation could be compared. Although the amounts of VFA produced were not significantly altered during period 1 (App. 5.2), the molar proportion of propionic acid was significantly higher ($p<0.05$) and that of total valeric acid ($p<0.01$) and the acetate: propionate

TABLE 7.5 Experiment 5a. Effects of monensin (334 nmol/d) and cobalt (47.6 nmol/d) supplementation upon mean VFA production/g DM digested (mmol) for vessels 1-4 given 7.22 g DM low cobalt hay(2)/d; s.d. of values are in parentheses.

		Period			
	transition	1	2	3	
Supplement	-	monensin	monensin + Co	monensin + Co	- Co
VFA					
Total VFA	6.72 (0.684)	7.92 (0.449)	6.81 (0.339)	7.57 (0.778)	7.89 (0.474)
Acetic acid	3.96 (0.318)	4.64 (0.329)	3.88 (0.193)	4.52 (0.509)	4.62 (0.269)
Propionic acid	1.28 (0.0968)	1.67 (0.207)	1.47 (0.101)	1.58 (0.0849)	1.63 (0.149)
Total butyric aci	0.830 (0.130)	0.948 (0.101)	0.870 (0.0622)	0.935 (0.163)	0.975 (0.0778)
Total valeric acid	0.656 (0.207)	0.539 (0.116)	0.584 (0.0523)	0.534 (0.0467)	0.682 (0.00636
Acetate: propionate	3.09	2.78	2.64	2.86	2.83
DM digested (g/d)	4.33 (0.0580)	4.19 (0.0585)	4.23 (0.0173)	4.07 (0.0354)	3.94 (0.0778)

ratio ($p<0.05$) lower (App. 5.3). Expression of VFA production in terms of DM digested revealed increases in all the VFA except total valeric acid (Table 7.5) and these increases were significant ($p<0.05$) for both propionic and total butyric acid production.

Total VFA and acetic acid production were significantly ($p<0.05$) decreased during period 2, compared to period 1, when Co was infused, while the proportion of total valeric acid was increased ($p<0.05$). VFA production /g DM digested was significantly decreased ($p<0.05$) for both total VFA and acetic acid (Table 7.5).

Withdrawal of the Co supplement from vessels 3 and 4 during period 3 was not associated with any changes in VFA production or proportions, either between periods or vessels; however, there was a significant difference ($p<0.05$) between the 2 pairs of vessels in the total valeric acid produced /g DM digested (Table 7.5) and the overall trend was for values to increase in the unsupplemented vessels.

Discussion

Resting values with unsupplemented hay (transition period)

Substantial carryover effects were seen to occur in vitamin B12 synthesis from the treatments of Experiment 4 (Figs. 7.1, 7.2). Therefore, the use of values from period 1 of Experiment 4 as baseline values in evaluating responses to monensin ± Co was justifiable. In contrast, VFA patterns during the transition period were considered to be representative of a roughage fermentation (Apps. 5.2, 5.3) and were used as an internal comparison.

Effects of monensin upon vitamin B12 synthesis (periods 1, 2 and 3)

Comparing the conversion efficiencies for Co into Cbl between periods 1 of Experiments 4 and 5 (4.13 ± 3.42 % and 4.86 ± 2.71 % respectively) indicated that there was no influence of monensin upon Cbl synthesis when the low cobalt hay was given, although three of the four values from Experiment 4 may have been low, due to the influence of the atypical fermentations. Furthermore, the high Cbl content of the washings (Table 7.4) and Cbl production (Table 7.3) from vessels 2 and 3 during this period indicated that carryover of Co and/or Cbl from the previous soil treatments may have occurred. The effect of any such carryover would be to overestimate conversion efficiencies.

A similar comparison for analogue synthesis (9.40 ± 9.00 % vs. 18.8 ± 2.84 %) suggested that the same carryover phenomenon may have affected the estimates of analogue synthesis, as responses in analogue production were less in those vessels (1 and 4) which had longer to equilibrate. Therefore, an inhibitory effect of monensin on vitamin B12 synthesis may have been masked in this phase of the experiment.

Values for the conversion efficiencies of supplementary Co into Cbl and analogues (0.367 ± 0.0783 % and 12.4 ± 2.40 % respectively) were lower than values for Experiment 2 (1.29 % and 15.7 % respectively) in which a low Co hay was also given. This resulted in a high analogue: Cbl ratio (33.8 vs. 12.2 in Experiment 2), which suggested an alteration in the vitamin B12 synthetic pathway. An

interesting contrast was evident in the efficiencies with which Co in the hay and supplementary Co were incorporated into Cbl and analogues in the presence of monensin. For the Co in hay, the conversion efficiency for Cbl synthesis was considerably higher (4.86 ± 2.71 % vs. 0.367 ± 0.0783 %); but for analogue synthesis the value was only 50 % greater (18.8 ± 2.84 % vs. 12.4 ± 2.40 %). While qualifications must be placed on the values for unsupplemented hay because of possible carryover effects, these would have increased the value for analogue synthesis more than that for Cbl synthesis as equilibration took longer. The absence of such a contrast in previous experiments, in which monensin was absent, is further indirect evidence of an effect of monensin on the partition of infused Co between Cbl and analogues.

Another unusual feature of Experiment 5 was the delay in attainment of equilibrium for analogue synthesis when Co was infused. Analogue production increased more than 3-fold between the 12th and 20th day of Co supplementation into vessels 1 and 2, while withdrawal of the Co supplement fron vessels 3 and 4 was followed by a 2-3 fold increase in analogue production. The sustained increases in analogue production might be explained in part by the delayed release of these compounds from microbial binding (Ford, 1958) in compartments 2 and 3. Alternatively, microbial stores of Co may have increased during the period of high Co supplementation. The slow decline in the Cbl content of the washings and Cbl production for vessels 3 and 4 was also very different from the initial depletion phases of

previous experiments, e.g. Experiment 2b in which Cbl output decreased by an average of 24 % in 48 h after withdrawal of Co. Cbl synthesis may have been enhanced by either stored Co or by interconversion and recycling of the Co from analogues. However, it was impossible to determine whether these exaggerated carryover effects were definitely attributable to the action of monensin.

Monensin is known to affect cation fluxes across eukaryotic membranes (Pressman, 1976) and while its influence upon prokaryotic membranes has not been studied, the effects upon bacteria have. Any effect of monensin upon vitamin B12 synthesis by the rumen flora may be due to its action as a bactericide, particulary against Gram-positive bacteria (Haney and Hoehn, 1967, cited by Wallace et al., 1981). Chen and Wolin (1979) found that monensin was an effective antibiotic against some of the known "vitamin B12 producers", such as Butyvibrio fibrosolvens and Ruminococcus flavefaciens (p 30), although against others it was ineffective (Selenomonas ruminantium) or its effect was ephemeral (Bacteroides ruminicola). Any toxic effects influential in this experiment, could have been compensated for by increased vitamin synthesis from unaffected species, e.g. Wallace et al., (1981) found the composition of flora in Rusitec to be altered when monensin was added, but total microbial numbers remained constant.

Effects of monensin on the ADMD of hay (periods 1, 2 and 3)

The inclusion of monensin was associated with a marked reduction in digestibility in all 4 cultures during period 1 (Fig. 7.3). These reductions were not reflected in

vitamin B12 synthesis. However, Experiments 1 and 3 had shown differences in digestibility with time, when a hay or barley was used, and these appeared to be divorced from the treatments employed. The present changes cannot therefore, automatically be attributed to the treatment which obtained. Nevertheless, the uniform decline to lower levels of ADMD than those obtained previously strongly suggested an inhibitory effect of monensin. This is consistent with the findings of Simpson et al. (1976) who showed in vitro cellulose digestion to be inhibited by monensin. Wallace et al. (1981), using monensin with a hay/concentrate diet in Rusitec, found a reduced digestibility to be accompanied by a reduced, initial rate of fibre digestion and that flora composition, but not numbers, had altered. Stanier and Davies (1981) found monensin to have a consistent inhibitory effect upon the digestion of a roughage/concentrate ration, that was presumably adequate in Co. Surprisingly, in Experiment 5 there was no reduction in either acetate production or acetate proportions in the VFA (Apps. 5.2, 5.3), even though acetate is an end product of cellulytic activity.

It is worth noting that in vivo trials have found digestibility to be unaffected (Dinius et al., 1976; Lemenager et al., 1978) or increased (Horton, 1979; Adams et al., 1981) when monensin is added to the diet. The latter result is considered to be due to an increased rumen retention time (Lemenager et al., 1978), a decreased dilution rate (Allen and Harrison, 1979) or a lowered feed intake (Lemenager et al., 1978), none of which occur with

vitro simulations such as Rusitec. Chen and Wolin (1979) drew attention to the ability of some microorganisms to adapt to monensin supplementation; therefore, alteration of the flora composition, in favour of monensin tolerant species, may have resulted in reduced digestibility.

Infusion of Co was associated with an increased digestibility, although it did not quite attain the initial values. The effect of Co was sustained in all vessels throughout period 2, but only incompletely during period 3; thereby suggesting that if it was an effect of the Co, it was only temporary.

The initial effect of Co on digestibility could arise from the stimulation of vitamin B12 synthesis and the slight delay in period 2 before ADMD values increased may have been the time required for vitamin B12 synthesis to increase or for the diffusion of substantial amounts of elemental Co into the feed matrix. Vitamin B12 is required for a number of microbial pathways (Table 1.4) and therefore its role as a coenzyme may have been influential, if only temporary. Alternatively, monensin is known to modulate trans-membrane fluxes of cations in eukaryotes (Pressman, 1976) and may have induced a Co deficiency in the microbiota during period 1 that was corrected in period 2. The dramatic decline in digestibility values during period 3, whether or not Co was supplemented, was possibly due to a readjustment of the microbial ecosystem that had been influenced by the addition of Co but that had adapted to its presence or alternatively, was the result of a slow accumulation of analogue forms which eventually inhibited coenzymic action.

VFA synthesis

Effects of monensin on VFA synthesis (period 1) Increases in the molar proportion of propionic acid have been found to occur *in vivo* for roughage diets supplemented with monensin (Dinius *et al.*, 1976; Richardson *et al.*, 1976), but not in Rusitec given a hay substrate (Wallace *et al.*, 1980). In Experiment 5 the elevation of propionic acid encountered was in accord with the role of monensin as a "propionate enhancer". The increases in VFA production /g DM digested that occurred in this experiment were also contrary to the results from Wallace *et al.* (1980) who used similar levels of monensin supplementation. Although their experiment was brief (4 d), the effects of monensin on VFA production in Experiment 5 were apparent after 4 d of period 1 and the conflicting results are difficult to explain.

Effects of cobalt supplementation upon VFA synthesis in the presence of monensin (periods 2 and 3) The drop in VFA production /g DM digested when Co was infused during period 2 was a result of an increase in digestibility and a decrease in VFA production. While VFA synthesis altered during this experiment (Apps. 5.2, 5.3) the changes throughout the experiment were not correlated with either vitamin B12 synthesis or digestibility.

Adaptation by the rumen ecosystem to monensin

Other workers who have studied monensin have found parameters to vary with time. The possible influence of adaptation has been recognised in *in vitro* work; Lemenager *et al.* (1978) found the depression of digestibility for monensin-adapted cultures to be less than that for unadapted cultures and monensin-adapted cultures have been used in other *in vitro* studies (Whetstone *et al.*, 1981). Wallace *et al.* (1981) encountered an upwards "drift" in methane and acetate production when Rusitec was given a hay/concentrate ration supplemented by monensin at levels one-fifth those used in Experiment 5. A temporary depression of digestibility in lambs (Poos *et al.*, 1979) following the inclusion of monensin was attributed to changes in the microflora.

If microbial adaptation to monensin occurs *in vivo* it is insufficient to prevent the additive improving feed conversion efficiencies in cattle fed either roughage- or concentrate-based diets for periods in excess of 2 months (Utley *et al.*, 1977; Wilkinson *et al.*, 1980; Adams *et al.*, 1981).

Conclusions

1. Enhancement of the molar proportion of propionic acid was achieved with a monensin supplement, even though digestibility was depressed.
2. An influence of monensin upon vitamin B12 synthesis, for unsupplemented and Co supplemented hay, posssibly via a toxic effect upon certain microbial species was suggested, but which had been masked by carryover effects from previous high Co inputs.

3. That monensin can drastically alter microbial metabolism was suggested by the variation in digestibility with time, an effect that was influenced by Co supplementation. The continued use of monensin drew attention to possible differences in the time required for stable production levels of the different forms of vitamin B12.

EXPERIMENT 5b A COMPARISON OF COBALT NITRATE AND COBALT EDTA AS SOURCES OF COBALT FOR VITAMIN B12 SYNTHESIS

Introduction

Chelated trace elements are applied to both soils and livestock with the objective of improving trace element uptake. EDTA is the most common chelate for Co and while its efficiency as a herbage treatment has been criticised, its inclusion in the diets of ruminants requires investigation. The low efficiency with which Co nitrate was utilised for Cbl synthesis in the previous experiments prompted a brief examination of the efficiency with which Co EDTA was used for this purpose. The addition of EDTA to the diets of ruminants has been shown to influence the distribution of trace elements in the gastrointestinal tract (e.g. Powell et al., 1967) and trace element chelates, including Co, are available commercially in both the British Isles and New Zealand for addition to the diet, without any published evidence to substantiate their advantages over simple inorganic salts.

Experimental procedure

The 4 Rusitec cultures from experiment 5a were continued, using the same low Co hay(2) given at 7.22 g DM/d and with monensin infused at the previous level (334 nmol/d). The Co supplementation of vessels 1 and 2 was continued at the same level as in Experiment 5a for a further 8 d (d 43-50) but the form was changed from the nitrate to an EDTA compound, containing 14 % Co by weight. Vessels 3 and 4 continued to receive no Co supplement. As in Experiment 5a, samples of the effluent and the washings were collected and the dilution rate was maintained at 0.75 /d.

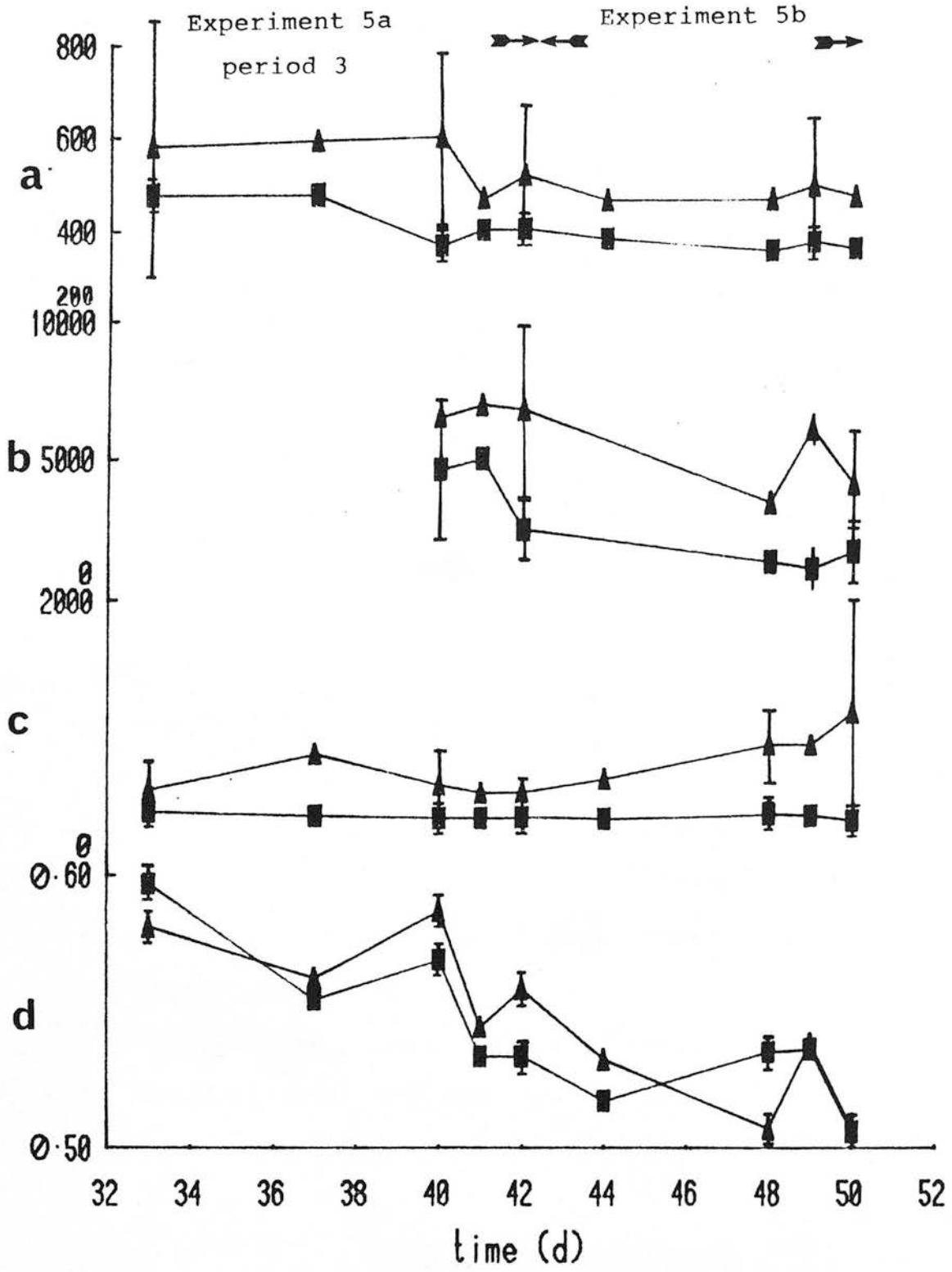


FIG. 7.4 Experiments 5a and 5b. Mean (± s.d.) values of a) Cbl production (pmol/d), b) analogue production (pmol/d), c) Cbl content of the washings (pmol), d) ADMD, in vessels 1 and 2 given 47.6 nmol Co/d, first as the nitrate (▲) and then as Co EDTA (▲) compared with the mean (± s.d.) for vessels 3 and 4 given no cobalt throughout (■). All vessels were given 7.22 g DM low cobalt hay(2) and 334 nmol monensin /d.

TABLE 7.6 Experiments 5a and 5b. Mean VFA production/g DM digested (mmol) when vessels were supplemented with 47.6 nmol Co as the nitrate/d during period 3 of Experiment 5a and with Co as EDTA during Experiment 5b. Vessels 3 and 4 had no Co supplement, but all 4 vessels were supplemented with monensin at 334 nmol/d and given 7.22 g DM low cobalt hay(2)/d (s.d. in parentheses, n=2).

	Expt. 5a		Expt. 5b	
	Supplement			
VFA	+ Co nitrate	- Co	+ Co EDTA	- Co
Total VFA	7.57 (0.778)	7.89 (0.474)	7.64 (0.297)	8.04 (0.403)
Acetic acid	4.52 (0.509)	4.62 (0.269)	4.47 (0.248)	4.74 (0.198)
Propionic acid	1.58 (0.0849)	1.63 (0.149)	1.67 (0.0778)	1.63 (0.127)
Total butyric acid	0.935 (0.163)	0.975 (0.0778)	0.909 (0.0474)	0.963 (0.0481)
Total valeric acid	0.534 (0.0467)	0.682 (0.00686)	0.617 (0.0941)	0.719 (0.0368)
DM digested (g)	4.07 (0.0354)	3.94 (0.0778)	3.74 (0.163)	3.80 (0.0212)

Results

Vitamin B12 synthesis

Neither Cbl or analogue production were enhanced by the use of the Co chelate; indeed there was a tendency for both to decline (Fig. 7.4). The elevated values for Cbl in the washings of the Co-supplemented vessels on d 48-50 were entirely due to an elevation of the values for vessel 1 which rose by 213 % during Experiment 5b, while that for vessel 2 remained constant. The analogue production from the unsupplemented vessels fell on average by 755 pmol/d (30 %) over the period d 43-50, to a level significantly ($p<0.01$) below that of period 3, however, Cbl production and the Cbl content of the washings remained constant (Fig. 7.4).

VFA synthesis and ADMD

There were no differences in VFA synthesis between the Co nitrate and Co-EDTA supplemented vessels, nor between supplemented and unsupplemented vessels (Apps. 5.2, 5.3). However, ADMD decreased and then stabilised in all vessels during Experiment 5b (Fig. 7.4), with a greater decrease in the Co-supplemented vessels. The only significant difference between the Co nitrate and Co EDTA supplement vessels in VFA production /g DM digested was for an increase ($p<0.05$) in propionic acid when the chelate was given and there were no differences between supplemented and unsupplemented vessels (Table 7.6).

Discussion

The advantage of chelated Co over inorganic sources would have to be large to cover the additional costs of the material. Although this experiment had only 2 replicates and was of relatively short duration, it seems unlikely that Cbl or analogue production could be sufficiently enhanced by using the chelated form of the Co supplement. The only doubt was raised by the large rise in Cbl levels of the washings for vessel 1, but it had given fluctuating results in Experiment 5a (Fig. 7.2). In view of the possibility of large carryover effects from the Co supplement used in Experiment 5a, which only became known after the completion of Experiment 5b, it was possible that Co-EDTA was used less efficiently than the Co nitrate, but that the effect was masked by the short time allowed for equilibration. However, as monensin is known to affect cation fluxes, it could be that an enhanced microbial absorption of the Co EDTA was slow in being reflected in production values.

The low digestibility of the hay during this period was probably due to the continuing effect of monensin additions. The fact that Co-EDTA was unable to counteract this inhibitory effect again suggests a low, rather than a high, biological availability in the light of the apparent response to Co nitrate during period 2 of Experiment 5a.

A reduced microbial absorption of Co EDTA would appear to be essential to justify the use of this chelate as a liquid phase marker in cattle (Haaland et al., 1982). Co given as the nitrate is incorporated into vitamin B12 and occurs

predominately in the solid phase of rumen contents and it seems likely that >15 % might be lost from the liquid phase. There is thus a further reason for investigating the availability of Co-EDTA to rumen microorganisms in the absence of monensin, something for which there was insufficient time in this investigation.

Conclusion

1. There was no evidence in this brief investigation that Co EDTA was utilised more efficiently than Co nitrate as a source of Co for Cbl or analogue synthesis; however, in view of the importance of improving the efficiency of Co utilisation, other chelates should be examined in the presence and absence of monensin.

CHAPTER 8

INTEGRATING DISCUSSION

In the course of this project it was not always possible to derive unqualified conclusions from individual experiments. However, as experience of Rusitec and vitamin B12 analysis accumulated, several important features became apparent. The main object of the study, namely to quantify vitamin B12 synthesis when rumen cultures were maintained *in vitro* under different dietary conditions, was achieved; but only when experiments are considered with regard to each other can their full value be appreciated.

Vitamin B12 assay techniques

The limitations of existing assays were comprehensively discussed in Chapter 1. The major problems concern the lack of specificity of the assay methods, whether microbiological or c.p.b. This lack of specificity becomes a major problem if the sample analysed is rich in analogues, e.g. rumen fluid, and even in body fluids only specific assays will establish the extent to which analogues are present. That the presence of analogues in blood is anticipated, has been demonstrated by the introduction of assay kits for Cbl that utilise cobinamide as a blocking agent to any nonspecific elements in the binder (Becton Dickinson U.K. Ltd.). Such concerns are relevant to the livestock industry and specific radioassays are now superseding microbial assays in veterinary laboratories (Wright *et al.*, 1982).

It is only during the course of the current studies that radioassays specific for Cbl have been developed, and then only for blood analysis. Consequently, values obtained by workers prior to 1978, purporting to be estimates of Cbl or total vitamin B12, will be unreliable whatever the material. Even the popular assay for Cbl using P.malhamensis is of questionable reliability and accuracy (p 60), due probably to inhibition of the Cbl response by analogues (Ford, 1959; Kamikubo and Hayashi, 1979). Such considerations cast doubt on both the quantitative and qualitative results of the vast majority of previous studies on ruminal vitamin B12 synthesis, whether microbial or c.p.b. assays were used.

In future, the wider use of assays specific for Cbl, such as the method developed during this study (p 84), will produce more reliable data. Validation of the assay for samples from Rusitec showed it to be unaffected by the addition of cobinamide, a common rumen analogue. Subsequent studies with a similiar technique applied to ruminant plasma samples have revealed excellent recoveries and reproducibility (N. F. Suttle, personal communication, 1983). Furthermore, the method was more sensitive and less than one-tenth the cost of the commercial kit with which it gave good agreement (Becton Dickinson U.K. Ltd.). Residual binding in bovine serum samples has been shown to vary between samples (Judson et al., 1982; Wright et al., 1982), but it was not a problem with the method developed here.

In view of the problems inherent in microbial assays, "specific" or otherwise, development of a rapid nonspecific

radioassay for total vitamin B12 would be valuable. This would require elucidation of the affinity of any nonspecific binder for different forms of the vitamin. As a result of the problems revealed by the Rusitec studies, methods for the separation and quantification of the analogues are now being investigated using HPLC techniques at the Moredun Institute. It should be noted that any nonspecific assay could not be validated if prior separation of the analogues was not achieved and the appropriate analogue used as a standard.

The thoroughness of the extraction phase of vitamin B12 analysis has received little attention and was recently called into question with regard to plasma samples (Gimseng, 1983). The extraction of vitamin B12 from digesta and other samples containing microorganisms may pose particular problems, particularly as the levels of stored, microbial vitamin B12 can be high (Oginsky, 1952; Kashket et al., 1962), and the extent to which this important source of the vitamin is available upon death of the cell will be of great importance to both the assay technique and the ruminant. In this study, as in most previous studies, the analyte was the vitamin B12 free in or extractable from the rumen sample. Clarification of the source of the vitamin B12 measured should be attempted in both rumen and abomasal samples; both will contain dead and lysed microorganisms, particularly the latter due to the acidic conditions of the abomasum. The proportions of vitamin B12 retained intracellularly in both Rusitec and in in vivo samples could be compared by suitable treatment of the microbes, e.g. sonication.

Rusitec as an experimental technique

The simplicity of operating Rusitec as a continuous rumen culture system over an extensive period was confirmed; but several questions were raised over its use in studies of rumen fermentation, particularly with regard to vitamin B12 studies.

Culture differences For both hay and barley substrates, differences between cultures were evident in vitamin B12 synthesis and other rumen parameters in all but one (Experiment 2) of the 5 experiments. Culture differences were evident as long-term alterations in VFA synthesis, e.g. Experiments 1, 3 and 4, and/or digestibility, e.g. Experiment 1. In Experiment 3, there were also short-term differences in VFA synthesis between cultures, i.e. the culture in vessel 3, and a change in the VFA pattern was associated with changes in vitamin B12 synthesis. Differences between cultures were particularly evident in vitamin B12 synthesis.

Individual differences in fermentation characteristics have been found previously in sheep given concentrate rations (Hodgson and Thomas, 1975). These workers found one of 2 fermentation patterns, high acetate: propionate ratio vs. low acetate: propionate ratio, to be produced and both animal differences and the previous feeding regime were suggested as possible causes. More recently, different fermentations have been found to occur with *in vivo* studies using sheep given silage (P. Thomas, personal communication, 1983). The culture differences shown in Rusitec are thus consistent with *in vivo* data, but they

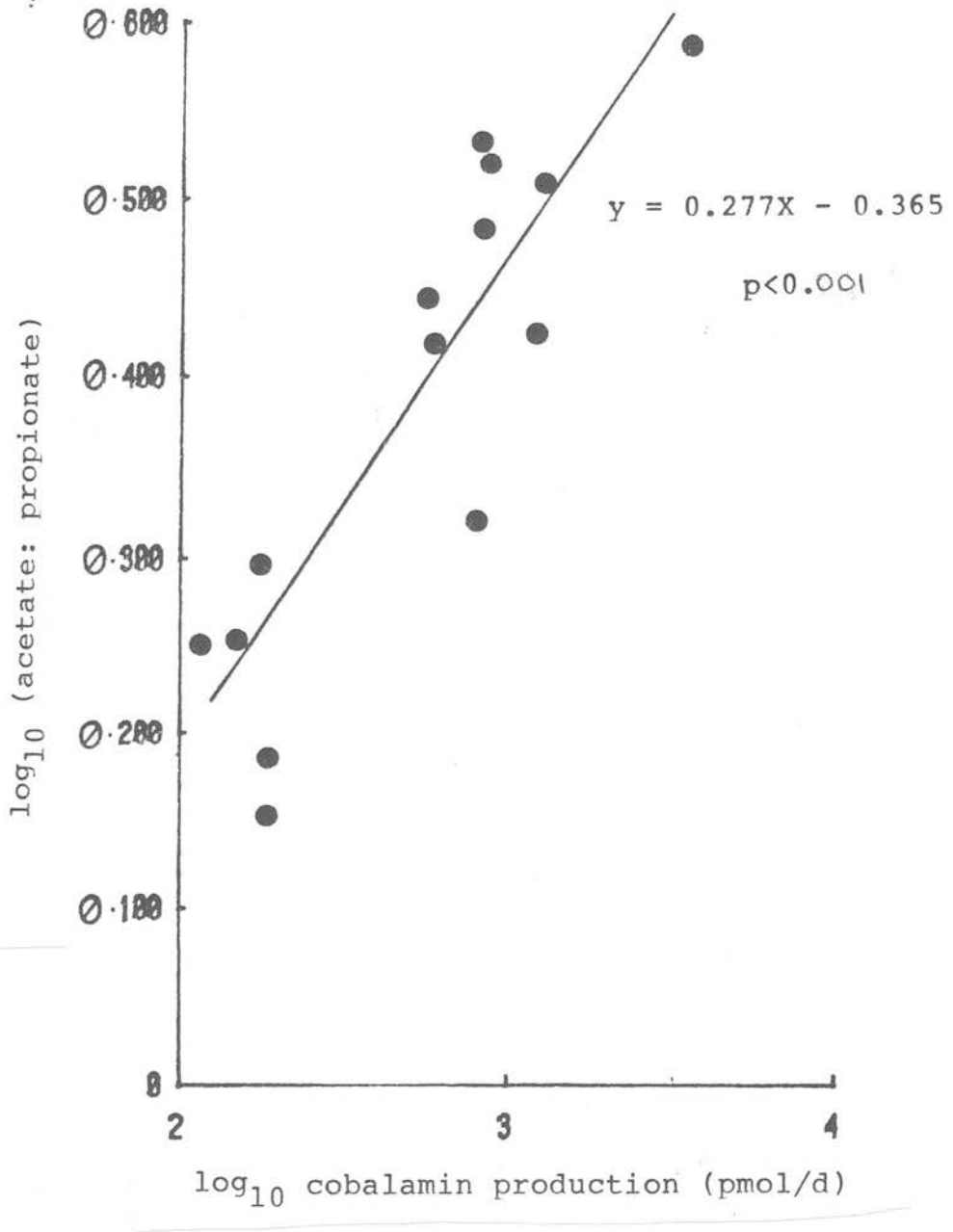


FIG. 8.1 The relationship between log cobalamin production and log acetate: propionate for the unsupplemented low cobalt hays (1) and (2) used in Experiments 1, 2, 3, and 4. The values for Experiments 1 and 2 were corrected (see p 208).

The presence of culture differences in Rusitec has implications for its use as a model for studies of rumen fermentation. As uniformity cannot be assumed, economies in the use of vessels, i.e. 1 vessel/treatment, and the use of consecutive treatments may give erroneous results. Previous workers who have used Rusitec may have been fortunate in not encountering such problems, but a more cautious approach is indicated. There are 3 alternatives.

1. The repetition of all experiments to ascertain if results were fortuitous.
2. The use of more vessels, e.g. 2 separate systems of 4 vessels, to try and account for culture differences. Replication would allow for the assessment of culture differences and covariance analysis might then be used to reduce their contribution to variation between subsequent treatments.
3. The use of a Latin Square design which accomodates both individual variation and carryover effects (Cochran and Cox, 1957).

Of these alternatives the third is the most reliable statistically, but the overall time required would be determined by the rates of response to, and the recovery from, treatments. For an experiment involving 4 treatments the duration would probably be in excess of 4 months, i.e. 18 d to achieve stability in vitamin B12 production for each treatment and a similar period to deplete levels between treatments.

The reintroduction of washings to maintain continuity of culture conditions is a problem unique to Rusitec and is very different from an in vivo rumen culture. This displacement of a proportion of the microbiota was reflected in the high, initial Cbl concentrations in the vessel fluid during the diurnal variation studies. Indeed, it was a good indication of the Cbl production potential for a particular vessel. The subsequent decline in Cbl concentrations illustrated the rapid rate of sequestration of organisms by the fresh substrate. However, it meant that estimates of vessel productivity from effluent samples would presumably be overestimated in comparison with an undisturbed culture. Colonisation of the new substrate would no doubt have occurred if these washings had not been returned and it would be worthwhile to examine the effects on fermentation, e.g. VFA synthesis, digestibility, that such a procedure would cause. By not returning the washings, the attainment of equilibrium of vitamin B12 levels during and after treatment may be accelerated.

Another feature of Rusitec is the maintenance of a near-neutral pH by the infusion of a buffer. The provision of barley to livestock can cause acidosis in the rumen, which may result in the depletion of vitamin B12-synthesising microorganisms. Therefore, if artificially maintaining the pH was influential, it would be more likely to have affected the barley rather than the hay fermentation.

TABLE 8.1 Experiments 2-5. Limits on periods of time (d) required for stabilisation of cobalamin and analogue synthesis from individual Rusitec cultures.

Expt.	Substrate	Cbl	Analogues
2a	hay(1)	5-11	⩾11
2b	hay(1) + Co	7-9	⩾9
2b	different hays (after + Co)	10->15	12->15
3	barley	4->16	⩾16
3	barley + Co	10->14	10->14
3	barley + Co	10->14	10->14
4	hay(2)	6-8	⩾8
4	hay(2) + soil	10->14	10->14
4	hay(2) (after + Co)		
	vessels 1 and 4	>21	>21
	vessels 2 and 3	>6	>6
5	hay(2) + monensin	10->12	10->12
5	hay(2) + monensin + Co	10->12	22->24
5	hay(2) + monensin (after + Co)	10->12	>10

Equilibration of vitamin B12 synthesis The time taken for vitamin B12 productivity to stabilise was shown to be important in determining treatment effects. This was particularly true in view of the high proportion of cultures that produced "atypical" fermentations, which often appeared to take longer to reach equilibrium. While the assessments of equilibrium were subjective, limits can be placed on the periods of time required for values representative of the treatment to be achieved (Table 8.1). Such values must be viewed with regard to previous treatments.

The equilibration period was dependent upon the substrate, and whether it was unsupplemented or supplemented with Co. When a low Co hay provided the initial substrate, Cbl synthesis in the cultures was stable after 5-11 d, whatever the inoculum, whereas for analogue synthesis the estimates were generally in the range 10-15 d. A greater affinity of analogues for microbial cell wall receptor sites in compartments 2 and 3 would delay equilibration of these compounds. When barley was used as the substrate there was a large variation in the time taken for Cbl production to equilibrate (4-16 d) and the analogue production took longer than 16 d. In that experiment the fermentation was unstable; this was probably due to the lower volume of the feed matrix. With soil supplementation, differences were found between the soils in the time required for equilibrium and have been discussed in Chapter 6. When monensin and Co were given as supplements in Experiment 5, the time required for analogue

production to equilibrate was apparently longer than had previously been encountered with either the hay or the barley substrate. No firm conclusion can be made regarding the influence of monensin in this, as the periods allowed earlier to attain equilibrium were generally shorter and therefore, less confidence can be placed in the earlier estimates of equilibration times.

Delays in equilibration of both Cbl and analogue production were sometimes marked after periods of Co supplementation than at other times, e.g. Experiment 2b. In Experiment 4, there were unexpected increases in analogue production after soil supplementation had ceased 18 d previously and they continued for 16 d. A similar occurence happened after the withdrawal of Co from the 2 vessels in Experiment 5. The continuing increase in vitamin B12 levels after periods of Co supplementation was unlikely to have been due simply to the capacity of microorganisms to store high levels of vitamin B12. Because the release rate should reflect the size of the store, the phenomenon must reflect further increases in the storage of vitamin B12 and this continued synthesis of vitamin B12 was almost certainly facilitated by Co sequestered during the previous periods of Co supplementation. Furthermore, it appeared that stored Co was used for analogue rather than Cbl synthesis. Microbial "storage" was shown to operate over a period of weeks (Experiment 4) and if persisting for longer in the field it may influence blood vitamin B12 levels long after a change of grazing or feed.

TABLE 8.2 Experiments 2 and 3. Three different measures of cobalamin production (pmol/d) by four Rusitec cultures.

A. Output values in Experiment 2 were the product of the cobalamin concentration in compartment 1 at 24 h and the effluent volume.

B. Production (area) values in Experiments 2 and 3 were derived from the area under the cobalamin vs. time curve, which was composed from diurnal samples.

C. Production values in Experiment 3 were determined by sampling the effluent accumulated during 24 h.

The substrates were hay(1) in Experiment 2 and barley in Experiment 3.

		A Output	B Production (area)	B: A
Expt. 2	Vessel			
- Co	1	374	851	2.28
	2	353	735	2.08
	3	361	746	2.07
	4	545	1260	2.31
+ Co	1	1070	1420	1.33
	2	715	967	1.35
	3	854	1090	1.28
	4	1150	1230	1.07
- Co	1	673	1490	2.21
	2	362	843	2.33
	3	596	978	1.64
	4	338	889	2.63

		C Production	B Production (area)	B: C
Expt. 3	Vessel			
- Co	1	879	950	1.08
	2	140	327	2.34
	3	322	344	1.07
	4	1340	1280	0.959
+ Co	1	918	1330	1.45
	2	307	595	1.94
	3	1640	1810	1.10
	4	1260	1260	1.00

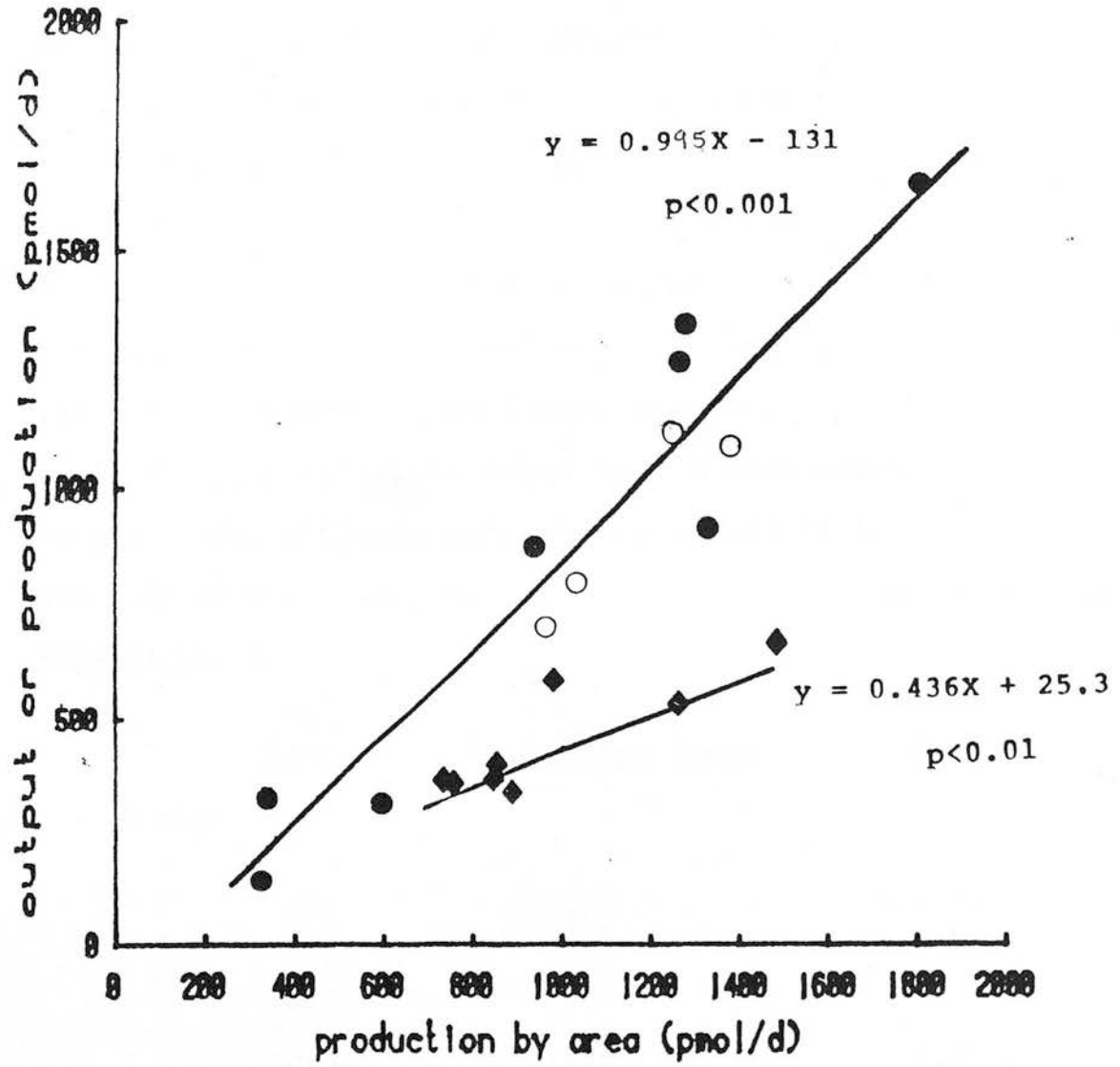


FIG. 8.2 The relationship of cobalamin production (pmol/d) to measures of:

1. Cobalamin output (pmol/d) by the unsupplemented hays (◆) and the cobalt supplemented hay(1) (○) during Experiment 2.

2. Cobalamin production (pmol/d) by the barley (●) when unsupplemented and cobalt supplemented during Experiment 3.

The subjective assessment of equilibrium is open to improvement, e.g. a statistical definition of stability, but would require more data from longer experiments. More replicates would also be required in view of the large variances associated with vitamin B12 production, something that did not occur with other rumen parameters, e.g. digestibility.

Productivity estimates from Rusitec

Three different methods were used to quantify the outflow of Cbl from Rusitec cultures and they sometimes gave different results (Table 8.2). In Experiment 3, there was close agreement between estimates obtained by area and by effluent analysis for unsupplemented and Co-supplemented cultures, with barley as the substrate (Fig. 8.2). Estimates by vessel fluid analysis and by area were in good agreement during periods of Co-supplementation in Experiment 2, while the former were only 43.6 % of the latter when no Co was given to the low Co hay(1) (Fig. 8.2). The difference was probably due to the the initial displacement of a proportionally larger Cbl-rich compartment 2 when the low Co hay was fed, which increased area but not vessel fluid (24 h) estimates. In making the following comparisons between experiments for efficiencies of Co incorporation into Cbl and analogues it was assumed that in Experiment 2 the values for unsupplemented hays were underestimated by a factor of 2.29, the reciprocal of 0.436 (the slope of the linear regression of output vs. production for the unsupplemented hays, Fig. 8.2).

TABLE 8.3 Experiments 2, 3 and 4. Conversion efficiencies (%) of Co into cobalamin and analogue synthesis plus the analogue: cobalamin ratios for different substrates, both supplemented and unsupplemented.

Figures in parentheses are values for Experiments 1 and 2 prior to multiplication by a correction factor of 2.29.

		Conversion efficiencies				Analogues: Cobalamin	
		Cobalamin		Analogues			
Substrate	Expt.	-Co	+Co	-Co	+Co	-Co	+Co
hay(1)	1	90.7 (39.6)	1.95	-	-	-	-
hay(1)	2	24.5 (10.7)	1.29	160 (69.8)	15.7	6.53	12.2
hay(1)	2	28.4 (12.4)	-	268 (117)	-	9.44	-
early ryegrass	2	6.64 (2.90)	-	69.9 (30.5)	-	10.5	-
late ryegrass	2	5.36 (2.34)	-	50.6 (22.1)	-	9.44	-
clover	2	4.49 (1.96)	-	51.8 (22.6)	-	11.5	-
hay(1)	3						
V 1 and 4		24.0	-	170	-	7.08	
V 2 and 3		4.84	-	137	-	28.3	
barley	3		0.551		3.8	-	6.90
V 1, 3 and 4		63.3	-	82.4	-	1.30	-
V 2		7.45	-	36.1	-	4.85	-
hay(2)	4						
V 1		9.21	-	22.1	-	2.40	
V 2, 3 and 4		2.43	-	5.16	-	2.12	
hay(2) + monensin	5	4.86	0.367	18.8	12.4	3.87	33.8

N.B. Values for less than all four vessels are included where fermentations differed between vessels.

Conversion efficiencies of endogenous and supplementary Co

The addition of trace elements to diets is a common agricultural practice and consequently there is a need to know how efficiently the element is used. Determination of the conversion efficiencies of Co in the hay and barley substrates into Cbl and analogues are given in Table 8.3, for Experiments 2 (corrected values), 3 and 4. Correction of analogue outputs was performed using the Cbl factor (2.29), as the same phenomenon should have been occurring with all vitamin B12 forms, although possibly to different extents depending upon affinities for microbial cell wall receptor sites, in compartments 2 and 3.

The conversion efficiencies for the 2 low Co hays differed substantially (Table 8.3). Excluding the results for Experiment 1, where Co contamination was suspected, hay(1) produced a mean efficiency of 25.6 ± 2.41 % (n=3), against 4.13±3.42 % for hay(2). The former was a better "quality" roughage (proximate analysis, East of Scotland College of Agriculture, Table 3.1) than hay(2). The values for hay(2) were closer to those of the ryegrass and clover hays used in Experiment 2b, so the values for the unsupplemented roughages did not correlate with their Co contents, indicating that factors other than Co content were influential in determining Cbl production. Intrinsic differences between roughages, other than Co, have been implicated in *in vivo* studies of vitamin B12 synthesis (Bigger *et al.*, 1976; Looney *et al.*, 1976). Having found differences between the levels of incorporation of Co into Cbl for different roughages in Experiment 2b, it would be illuminating to examine the efficiency with which supplementary Co to these substrates was utilised in vitamin B12 synthesis.

More care has to be adopted with the values for analogue synthesis because of the occurrence of values in excess of 100 %, the significance of which have been mentioned previously. However, it is worth noting that Hedrich et al. (1973) found an efficiency of Co incorporation into analogues of 80.4 % for a predominately roughage diet, which was deficient in Co. Although the elevated results may have been obtained because equilibrium had not been achieved, they show the same pattern as that for Cbl, with the low Co hay(1) having the highest efficiency of incorporation into analogues.

For barley, the efficiency of incorporation of Co into Cbl for 3 of the 4 cultures was greater than that for any of the hays and provided no evidence that Co so supplied was less likely to be incorporated into Cbl than if supplied in a roughage. The efficiency of analogue synthesis from the Co in barley was within the range of values for the hays. The overall pattern was for a higher proportion of the total vitamin B12 synthesised from the barley substrate to be in the form of Cbl than for the low Co hay(1), whether or not atypical cultures were considered.

The efficiencies of Cbl synthesis when the hay and barley of Experiments 2 and 3 were supplemented with Co were greatly reduced from the values of the unsupplemented substrates (Table 8.3). Both Gawthorne (1970a) and Smith and Marston (1970a) have found, using roughage diets in vivo, a decrease in the conversion efficiency of Co incorporation into Cbl, when inorganic Co was provided, in rumen fluid and rumen contents respectively. However,

Hedrich et al. (1973) found that supplementary Co was used more efficiently than Co in a deficient feed, when ruminal production was measured in vivo by sampling the duodenal contents. Interestingly, there were substantial decreases, with both hay and barley, in the proportion of Cbl in the total vitamin produced when supplementary Co was provided in Rusitec, but the proportion of Cbl was greater with barley than with hay. These results were in contrast with the work of Sutton and Elliot (1972) who found a concentrate ration to produce a smaller proportion of Cbl than a roughage ration when both were supplemented with Co.

It was also possible in this project to estimate the efficiency of incorporation of supplementary Co into analogues, using Co-deficient hay and barley substrates. Although both values were greatly reduced from the unsupplemented results the reduction for the barley was far greater (ca. 20-fold vs. 8-fold).

When soils provided the supplementary Co, neither the total or acetate-extractable Co concentrations in the soil were related to Cbl production. The efficiencies with which total Co was incorporated into Cbl were closer to the values for inorganic Co than those for the unsupplemented hays, but they did vary between soils.

With monensin present, the incorporation of herbage Co into Cbl was achieved with approximately the same low efficiency as that for the unsupplemented hay(2) in Experiment 4; the efficiency of analogue production was approximately 4-fold greater than that for Cbl production.

Upon supplementing with Co, both values decreased but that for Cbl was reduced more than the analogue value, so producing an analogue: Cbl ratio far in excess of the previous values from Rusitec. The difference may reflect a shift in the microbial population from acetate to propionate producers, the former being more dependent upon and richer in Cbl.

The gross differences between the utilisation of plant and supplementary Co, if they are found in vivo will affect the attempts to define Co deficiency in terms of the dietary Co concentration. The most recent predictions (ARC, 1980; COSAC, 1982), placing the dietary requirement of sheep and cattle at 0.11 mg Co/kg DM (= 1870 nmol Co/kg DM), drew upon studies involving supplementary Co. If supplementary Co is utilised less effectively than plant Co in the synthesis of Cbl then the requirements would be lower if the entire dietary supply of Co was provided in the herbage. Current experiments at the Moredun Research Institute have shown that mature ewes can subsist on Co-deficient barley or hay, containing 0.02 and 0.05 mg Co/kg DM respectively (340 and 849 nmol Co/kg DM respectively), for at least 5 months (N. F. Suttle, personal communication, 1983). Therefore, accurate determinations of Co requirements should be made with feedstuffs as the principal Co source.

There is however a possibility that the Rusitec gives an underestimate of the efficiency with which rumen microbes utilise supplementary Co for Cbl synthesis in vivo. In Rusitec, none of the Co nitrate supplements appeared to

stimulate Cbl synthesis sufficiently to reach the requirement. The percentage increase required to achieve adequacy was assumed to be

$$\frac{\text{ARC requirement}}{\text{Co content of the substrate}} \times 100\ \%$$

which gave values of 225, 344 and 1010 % for hay(1), hay(2) and the barley respectively. The maximum response obtained was only 65.0 % in any of the experiments, yet the highest Co supplements employed were 4-fold greater than the recommended dietary levels. Evidence for the feed matrix being the major region of vitamin B12 synthesis is substantial and the most probable reason for a reduced synthesis of Cbl from infused Co, when compared to herbage Co, was a reduced supply of Co for the microorganisms in compartments 2 and 3. This may have occurred by adsorption of the infused Co onto the vessel structure, e.g. perspex walls, polystyrene container, or more probably because of the limited rate at which Co moves from the large compartment 1 into the smaller but crucial compartment 2.

The distribution of elemental Co in Rusitec should be investigated in future work, but the development of a sensitive, analytical technique for Co is a prerequisite. At the present time, the best prospects would appear to reside in the chemiluminescent technique of Montano and Ingle (1979).

Rusitec as a model of the rumen

The exaggerated distinction between compartments 1, 2 and 3 in Rusitec has certain advantages in that it allows the major site of vitamin B12 synthesis to be indicated and quantified if necessary. On reflection, it would have been interesting to have sampled the microflora of both bags and to have so assessed the full size of the Cbl store in compartment 2. The gross compartmentation in Rusitec may limit its usefulness in other nutritional contexts, e.g. Czerkawski and Breckenridge (1982) found that urease production was stimulated to a greater extent by a continuous infusion of urea rather than by a single daily addition.

The inclusion of soil in an *in vitro* system such as Rusitec may not adequately simulate the *in vivo* situation. The distinct differences between soil types, i.e. Cbl production, recovery of Co, recorded in Rusitec suggest that the role of soil in the rumen warrants further study, particularly with regard to the degree of retention and the rate of Co release. Although soil particles are retained in the reticulum, it would be surprising if there were not differences in the size of particles retained and the extent of retention. If 50 % of all ingested soil were retained in the reticulo-rumen, as it was in Rusitec cultures, the organ would soon become impacted. Nevertheless, the uptake of soil could be important on pastures where herbage production is low or which are overstocked, e.g. the yearly variations in Co-deficiency.

Vitamin B12 analogues were considered to have a major influence upon Cbl production from the rumen ecosystem. Their presence was considered to regulate the distribution (i.e. time required to attain equilibrium) and the utilisation of Cbl by microorganisms and a similar role would be expected *in vivo*. Therefore, any ration that encourages their production may have a detrimental effect upon microbial metabolism and ultimately upon the growth of the host. Analogue interference may be in any one of 3 ways.

1. Alteration of microbial metabolism and hence the end-products of digestion.
2. By binding to IF and reducing Cbl absorption (Kolhouse and Allen, 1977a).
3. By absorption into the host and the subsequent blocking of Cbl functions in host tissue, e.g. the postulated link between Cbl and the low milk-fat syndrome.

The culture differences that occurred in Rusitec may happen *in vivo* and might explain the variations in the occurrence of Co deficiency that have been encountered, as these culture differences were found to occur for both Co deficient and Co adequate diets. Differences in vitamin B12 synthesis caused by alterations in the rumen fermentation would affect levels of Cbl in the blood. Furthermore, such "atypical" fermentations may affect the digestive end-products, and hence the metabolic load. In such cases, the practical implications of "atypical" fermentations would be important, particularly with the increase in cereal-based rations and the resultant

propionate fermentation. A shift in the fermentation pattern from acetate to propionate would increase the requirement for Cbl in the liver and could induce a functional deficiency in the host.

Future work

This study investigated various aspects of the problems of Co deficiency as they relate to the fermentation of digestion. Future studies could follow up these results in a number of ways.

1. Validation of the analytical techniques for analogues needs to be rigorously undertaken. This study has prompted elucidative work at the Moredun Institute, the results of which bode well for future work.

2. The metabolic requirement of microorganisms for both Cbl and analogues and their relationship with the rumen fermentation demand a larger study, particularly with regard to the instigation and control of abnormal fermentations.

3. The presence of analogues in ruminant body fluids needs to be quantified and subsequent experiments should investigate, both *in vitro* and *in vivo*, the possibility of such compounds blocking Cbl-dependent pathways.

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APPENDICES

APP. 2.1 Experiments 2a and 2b. Cobalamin outputs (pmol/d) from the cultures in Rusitec vessels 1-4, with analogue outputs (pmol/d) in parentheses.

		Vessel			
		1	2	3	4
Day	Expt.				
1		-	2930	1230	2110
3		2140	3920	2150	3890
5	2a	771	1170	588	719
7		296(7220)	545(10600)	355(6190)	737(9640)
9		392(9310)	341(6960)	396(10200)	668(10100)
11		435(4820)	173(1330)	333(1800)	231(-)
13		497	345	264	657
15		592	308	418	602
17	2b	701	295	483	903
19		851	611	757	1620
21		1280(10800)	818(8610)	950(10600)	688(7780)
23		1230(9200)	605(10500)	837(10000)	441(5150)
25		1350(4520)	505(10800)	904(10000)	654(10800)
27		1010	307	904	437
29		822	119	668	331
31		706	175	159	200
33	2b	701	328	472	422
35		644	396	720	254
37		531(5360)	358(2900)	433(3770)	287(4400)
39		388(3860)	279(3070)	382(5310)	227(1800)
40		494(4160)	522(6210)	313(1600)	173(1740)

APP. 2.2 Experiments 2a and 2b. Digestibility of feeds in Rusitec vessels 1-4. ADMD values with AOMD values in parentheses.

	Vessel			
	1	2	3	4
Substrate				
Low cobalt hay	0.625 (0.624)	0.627 (0.624)	0.631 (0.629)	0.629 (0.627)
Low cobalt hay + Co	0.625 (0.627)	0.620 (0.601)	0.617 (0.622)	0.633 (0.639)
Different hays	0.644 (0.641)	0.762 (0.762)	0.751 (0.747)	0.767 (0.769)

APP. 3.1 Experiment 3. Cobalamin production (pmol/d) from the cultures in Rusitec vessels 1-4, with analogue production (pmol/d) in parentheses.

		Vessel			
		1	2	3	4
Day	Period				
5		640(6440)	207(5450)	137(3110)	1180(7250)
6	Initial	543(9260)	154(3442)	236(8710)	1280(2760)
8		1180	222	204	1380
10		698	135	132	817
11		615	102	91.8	793
13	1	627	119	163	1470
15		1130	161	481	1200
18		738(3220)	113(4670)	1330(873)	959(9582)
21		745(2400)	83.4(1660)	1080(307)	651(3290)
23		613(886)	91.8(465)	691(1570)	553(728)
25		641	84.0	624	474
27		745	95.4	1430	524
29		764	152	1240	465
31	2	665	168	1470	460
33		570(1050)	173(2190)	1740(3230)	557(1530)
35		873(1560)	225(1770)	1070(3190)	541(1230)
37		926(2020)	239(1860)	1740(4110)	708(2060)
39		994	321	1360	626
41		1040	300	1330	775
43		832	243	1790	941
45	3	704	232	1840	1060
47		767(2580)	360(1610)	1780(5260)	1270(4940)
49		918(4320)	343(3100)	1520(5200)	1330(4750)
51		1070(4420)	217(2110)	1630(6480)	1190(5520)
54		1000	335	2120	1700
55	4	692(557)	341(599)	1960(2300)	1080(1850)
56		827(691)	324(288)	2300(3090)	1550(2960)
57		727(686)	324(288)	2150(4650)	- (-)

APP. 3.3 Experiment 3. VFA production (mmol/d) and ADMD during periods 1-4. Each value is the mean of three results, unless indicated.

		Vessel			
	Period	1	2	3	4
VFA					
Total VFA	1	43.7	42.9*	38.2	40.7
	2	40.3	35.6	46.4	39.3
	3	40.5	40.7	40.6	39.5
	4	37.7	37.9	41.7	44.3
Acetic acid	1	13.4	13.3*	12.8	12.8
	2	14.8	10.7	17.0	13.2
	3	15.3	12.6	15.4	14.5
	4	14.3	13.0	16.9	18.4
Propionic acid	1	14.7	20.3*	12.4	12.8
	2	11.0	10.9	14.1	13.8
	3	12.4	15.1	14.0	13.2
	4	11.7	12.6	13.2	13.3
Total butyric acid	1	12.1	11.4*	9.95	11.9
	2	9.57	9.36	10.3	8.31
	3	8.92	9.04	7.87	7.43
	4	8.44	8.47	8.17	8.17
Total valeric acid	1	3.44	5.10*	3.07	3.20
	2	5.00	4.53	5.00	4.09
	3	3.84	4.09	3.29	4.24
	4	3.30	3.81	3.45	4.49
ADMD	1	0.875	0.873*	0.870	0.875
	2	0.881	0.886	0.883	0.877
	3	0.882	0.882	0.886	0.876
	4	0.878	0.879	0.882	0.872

* = mean of two results

APP. 4.1 Experiment 4. Cobalamin production (pmol/d) from vessels 1-4. Figures in parentheses are values for analogue production (pmol/d).

		Vessel			
		1	2	3	4
Day	Period				
2		4420	3920	2100	3330
4	1	1340(6790)	634(3180)	380(3630)	677(3900)
6		555(2130)	187(1950)	113(351)	138(66.0)
8		546(1320)	108(59.0)	113(383)	211(484)
11		683	105	101	322
13		882	98.2	117	578
15	2	1240	263	300	731
17		859	285	269	680
18		779(2270)	320(447)	307(5880)	413(775)
21		2540(8680)	256(1490)	223(5380)	813(5460)
22		2570(7490)	293(2350)	182(10800)	709(5010)
26	3	534	195	251	207
27		684	222	140	184
30		1040	430	390	143
31	4	1180(2200)	948(3090)	578(3750)	162(916)
36		594(1410)	824(2730)	825(3380)	164(674)
37		818(1690)	882(3100)	915(2460)	154(786)
40		1200(2100)	541(3060)	521(2990)	196(2160)
42	5	1590(4410)	616(3360)	533(3230)	211(2540)
43		1560(2570)	678(3380)	572(3660)	181(2770)

APP. 4.2 Experiment 4. VFA molar proportions of the total VFA (%) and the acetate: propionate ratio during periods 1-5. Each value is the mean of three results.

		Vessel			
		1	2	3	4
VFA	Period				
Acetic acid	1	58.4	49.0	48.3	50.4
	2	43.2	56.9	58.2	61.4
	3	56.3	52.4	55.1	50.6
	4	61.8	60.2	59.7	52.3
	5	60.0	60.3	58.6	57.5
Propionic acid	1	21.3	27.4	27.1	25.6
	2	27.6	19.9	19.7	17.2
	3	19.6	22.1	21.3	22.4
	4	16.9	20.4	20.7	21.1
	5	19.1	19.0	20.8	17.4
Total butyric acid	1	12.9	14.0	13.5	13.9
	2	19.2	14.1	11.4	12.8
	3	16.1	15.8	14.4	16.0
	4	13.7	10.8	10.9	13.6
	5	13.7	11.5	10.9	13.1
Total valeric acid	1	7.24	9.64	10.7	9.93
	2	9.64	8.74	10.3	8.62
	3	9.30	11.1	8.63	8.44
	4	7.31	8.41	8.57	12.4
	5	7.25	9.24	9.79	12.2
Acetate: propionate	1	2.77	1.80	1.79	1.98
	2	1.57	2.86	2.95	3.57
	3	2.87	2.37	2.59	2.26
	4	3.66	2.95	2.88	2.48
	5	3.14	3.17	2.82	3.30

APP. 4.3 Experiment 4. VFA production (mmol/d) during periods 1-5.

Each value is the mean of three results.

		Vessel			
		1	2	3	4
VFA	Period				
Total VFA	1	28.6	22.0	26.9	26.7
	2	19.5	31.8	35.2	29.6
	3	31.2	21.5	30.0	26.9
	4	32.7	30.2	26.6	29.0
	5	26.9	27.7	28.3	33.3
Acetic acid	1	16.8	10.8	13.0	13.5
	2	8.38	18.1	20.5	18.3
	3	17.7	11.3	16.5	13.6
	4	20.2	18.2	15.9	15.1
	5	16.1	16.7	16.6	19.1
Propionic acid	1	6.06	6.00	7.26	6.81
	2	5.39	6.35	6.95	5.04
	3	6.02	4.74	6.39	6.02
	4	5.56	6.03	5.43	5.86
	5	5.16	5.25	5.89	5.77
Total butyric acid	1	3.69	3.07	3.63	3.70
	2	3.75	4.48	4.04	3.75
	3	4.94	3.39	4.27	4.30
	4	4.59	3.20	2.84	3.79
	5	3.70	3.19	3.08	4.36
Total valeric acid	1	2.07	2.12	2.87	2.65
	2	1.88	2.78	3.62	2.55
	3	2.90	2.38	2.59	2.27
	4	2.39	2.54	2.28	3.59
	5	1.95	2.56	2.77	4.06

APP. 5.1 Experiments 5a and 5b. Cobalamin production (pmol/d) from vessels 1-4 during all periods of Experiments 5a and 5b.

		Vessel			
		1	2	3	4
Day	Period				
	Expt. 5a				
3		1200	541	521	196
5	trans.	1590	616	533	211
6		1560	678	572	181
7		2010	491	566	202
8		1380	430	324	208
10		807	252	202	141
13	1	717	227	460	375
16		532	296	266	169
17		569	355	189	150
18		455	185	192	123
21		497	244	256	206
24		629	293	320	267
28	2	704	363	365	277
29		760	422	406	382
30		646	412	451	391
33		766	395	466	487
37		760	424	409	545
40	3	729	475	363	375
41		559	383	381	424
42		626	413	416	393
44		522	411	402	363
48	Expt. 5b	468	-	334	377
49		601	392	391	359
50		531	415	371	349

APP. 5.2 Experiments 5a and 5b. VFA production (mmol/d) during Experiments 5a (periods 1-3) and 5b. Each value is the mean of three results.

		Vessel			
		1	2	3	4
VFA	Period				
Total VFA	trans.	26.9	27.7	28.3	33.3
	1	33.1	33.5	35.1	31.0
	2	29.2	26.8	30.2	28.8
	3	32.8	28.7	29.3	32.8
	4	28.4	28.6	31.7	29.3
Acetic acid	trans.	16.1	16.7	16.6	19.1
	1	20.0	19.7	20.5	17.5
	2	16.8	15.3	17.2	16.3
	3	19.7	17.0	17.2	19.2
	4	16.8	16.5	18.6	17.4
Propionic acid	trans.	5.16	5.25	5.89	5.77
	1	6.98	7.51	7.65	5.75
	2	6.18	5.82	6.79	5.99
	3	6.64	6.20	5.88	6.90
	4	6.24	6.20	6.56	5.82
Total butyric acid	trans.	3.70	3.19	3.10	4.36
	1	4.21	3.56	3.70	4.41
	2	3.94	3.30	3.70	3.77
	3	4.22	3.35	3.57	4.09
	4	3.41	3.37	3.80	3.51
Total valeric acid	trans.	1.95	2.56	2.77	4.06
	1	1.60	2.23	2.57	2.61
	2	2.23	2.36	2.57	2.71
	3	2.29	2.05	2.66	2.70
	4	1.99	2.63	2.84	2.62

APP. 5.3 Experiments 5a and 5b. VFA molar proportions of the total VFA (%) and the acetate: propionate ratio during Experiments 5a (periods 1-3) and 5b. Each value is the mean of three results.

		Vessel			
		1	2	3	4
VFA	Period				
Acetic acid	trans.	60.0	60.3	58.6	57.5
	1	60.4	58.9	58.3	56.5
	2	57.7	57.1	56.9	56.7
	3	60.0	59.4	58.7	58.5
	4	59.2	57.4	58.6	59.4
Propionic acid	trans.	19.2	19.0	20.8	17.4
	1	21.1	22.4	21.8	18.6
	2	21.2	21.7	22.5	20.9
	3	20.2	21.7	20.1	21.0
	4	22.0	21.8	20.7	19.9
Total butyric acid	trans	13.7	11.5	10.9	13.1
	1	12.8	10.6	10.5	14.3
	2	13.5	12.4	12.2	13.1
	3	12.9	11.7	12.2	12.5
	4	12.0	11.8	11.9	12.0
Total valeric acid	trans.	7.25	9.24	9.79	12.2
	1	4.83	6.66	7.32	8.42
	2	7.64	8.81	8.51	9.41
	3	6.98	7.14	9.08	8.23
	4	7.01	9.20	8.96	8.94
Acetate; propionate	trans.	3.14	3.17	2.82	3.31
	1	2.86	2.63	2.67	3.04
	2	2.72	2.63	2.53	2.71
	3	2.97	2.74	2.92	2.79
	4	2.69	2.63	2.83	2.99